Ligand-induced selective signalling at the gonadotrophin releasing hormone receptor

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DECLARATION

I declare that this dissertation is the result of my own work, and that apart from the expected guidance of my supervisors, I have received no additional assistance. Furthermore, no portion of this work has been submitted in the past, or is being, or is to be submitted for a degree at this university or any other university.

Marla C. Coetsee
January, 2008

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LIST OF ABBREVIATIONS

7TM	seven transmembrane
AC	adenylate cyclase
ADP	adenosine diphosphate
AGS	activator of G protein signalling
Akt/PKB	acutely transforming/ protein kinase B
ANOVA	analysis of variance
AR	adrenergic receptor
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
B_{max}	maximum binding
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CCP	clathrin-coated pit
Cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CHO	Chinese Hamster Ovary
CK2	casein kinase 2
COS	African green monkey kidney (cells)
CTX	cholera toxin
DA	dopamine
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EC_{50}	median (or half-maximal) effective concentration
ECL	extracellular loop
EDTA	ethylenediamine tetraacetic acid
EGF(R)	epidermal growth factor (receptor)
E_{max}	maximal agonist-stimulated IP production
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ETCM	extended ternary complex model
FCS	foetal calf serum
FM	functional domain
FRET	fluorescence resonance energy transfer
FSH	follicle stimulating hormone
G protein	guanosine nucleotide (GTP) binding protein
g	acceleration due to gravity
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GEF	guanosine nucleotide exchanger
GH_3	clonal pituitary cell
GIRK	G protein-activated inwardly rectifying K^+ channel
GnRH	gonadotrophin releasing hormone
GnRHR	GnRH receptor (type I unless otherwise stated)

GPCR	G protein coupled receptor
GRIN	G protein-regulated inducer of neurite growth
GRK	G protein-coupled receptor kinase
GST	glutathione s-transferase
GTP	guanosine triphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
GTPase	guanosine triphosphatase
H8	helix 8
HA	haemagglutinin
H-bond	hydrogen bond
HEK293	human embryonic kidney 293
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HH	hypogonadotrophic hypogonadism
IC_{50}	concentration giving 50% of maximal inhibition
ICL	intracellular loop
IFN-γ	interferon-gamma
IGF-I(R)	insulin-like growth factor type I (receptor)
IL2R	interleukin-2 receptor
IN3	(2*S*)-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxo-ethyl]- 2(3,5dimethylphenyl)-1*H*-indol-3-yl]-*N*-(2-pyridin-4-ylethyl) propan-1-amine
iNOS	inducible nitric oxide synthase
IP	inositol phosphate
IP_3	inositol 1,4,5-trisphosphate
ITIM	immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinase
JNK	c-Jun-aminoterminal kinase
K^+	potassium ions
K_d	dissociation constant
kDa	kilodalton
KSR-1	kinase suppressor of Ras-1
LARG	Leukemia-associated RhoGEF
LH	luteinising hormone
LiSS	ligand-induced selective signalling
MAPK	mitogen-activated protein kinase
MD	molecular dynamics
Mg^{2+}	magnesium
MIP-1β	macrophage inflammatory protein-1 beta
MMP	matrix metalloprotease
MOI	multiplicity of infection
MTSEA	methanethiosulphonate
NE	norepinephrine
NF-κB	nuclear factor-kappa B
NHE	Na^+/H^+ exchanger
NIS	no information supplied
NMR	Nuclear Magnetic Resonance
NP40	Nonidet P-40
NS	non-stimulated (control)
OH	hydroxyl
p	probability
PAGE	polyacrylamide gel electrophoresis

PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDGF	platelet derived growth factor receptor
PDZ	PSD-95, Discs-large and ZO-1 domain
PEG	polyethylene glycol
PI3K	phosphotidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKD	protein kinase D
PLC	phospholipase C
PLD	phospholipase D
PP2A	serine/threonine protein phosphatase 2A
PRL	prolactin
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PTX	pertussis toxin
Q	signalling efficiency
RAMP	receptor activity-modifying protein
RGS	regulator of G protein signalling
RTK	receptor tyrosine kinase
RTP	receptor tyrosine phosphatase
SAPK	stress-activated protein kinase
SCL60	HEK293 cells stably transfected with the rat GnRH receptor
SDF-1α	stromal cell-derived factor-1 alpha
SDS	sodium dodecyl sulphate
SDSL	site-directed spin labelling
SE	standard error
Sf21	*Spodoptera frugiperda* 21 (insect cells)
SFK	src family of protein tyrosine kinase
SH2	src homology 2 (domain)
SH3	src homology 3 (domain)
SHP-1	SH2 domain-containing phosphatase 1
SHP-2	SH2 domain-containing phosphatase 2
SM	structural motif
SPA	Scintillation Proximity Assay
STAT	signal transducer and activator of transcription
T4L	T4-lysozyme
TCM	ternary complex model
TIMP	tissue inhibitors of metalloproteinase
TM	transmembrane
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
TSHR	thyrotropin receptor
uPA	urokinase-type plasminogen activator
UT	untransfected

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ABSTRACT

The pituitary gonadotrophin releasing hormone (GnRH) receptor regulates reproduction by activation of $G_{q/11}$ proteins. In contrast, GnRH receptors at extrapituitary sites induce anti-proliferative effects that do not correlate with $G_{q/11}$ activation. We propose that the two endogenous ligands, GnRH I and GnRH II, and certain antagonists selectively activate distinct signalling pathways by stabilisation of distinct active conformations of the GnRH receptor, a concept termed ligand-induced selective signalling (LiSS). This dissertation has investigated LiSS at the GnRH receptor using several approaches.

The sequences of GnRH I and II differ in positions 5, 7 and 8. I investigated the interaction of position 5 of GnRH I and GnRH II with $Tyr^{6.58}$ of the receptor. Compared with the Leu and Ala mutants, the $Tyr^{6.58}$Phe mutant had higher affinity for native GnRHs, but not Ala^5-substituted GnRHs, suggesting that Tyr^5 of GnRH I and His^5 of GnRH II interact with $Tyr^{6.58}$ by aromatic interactions. Our molecular models show that GnRHs interact with distinct rotamer conformations of $Tyr^{6.58}$. This is supported by the $Tyr^{6.58}$Leu receptor, which makes compensatory interactions that improve binding affinity and receptor activation for GnRH II, but not GnRH I, compared with the $Tyr^{6.58}$Ala receptor. Together these results suggest that GnRHs stabilise distinct receptor active conformations.

To identify the most proximal signalling proteins that mediate GnRH receptor-dependent anti-proliferative effects, I established a range of [^{35}S]GTPγS binding assays. I confirmed that the GnRH receptor activates $G_{q/11}$, but in contrast to previous proposals, my results show that the GnRH receptor cannot directly activate G_i. I subsequently identified a novel GnRH receptor signalling partner, the SH2 domain-containing phosphatase 2 (SHP-2). I propose that SHP-2 mediates the anti-proliferative effects of the receptor. I show that the SHP-2 pathway is activated independently of $G_{q/11}$ and suggest that signalling occurs by a direct interaction of SHP-2 and src with the GnRH receptor. Furthermore, this pathway is activated by a classical $G_{q/11}$ antagonist or by $G_{q/11}$-uncoupled GnRH receptor mutants.

My results provide convincing evidence supporting LiSS at the GnRH receptor and may facilitate development of therapeutics with increased signalling specificity at this receptor.

1 Chapter 1: Literature Review

1.1 Physiological and therapeutic roles of GnRHs and GnRH analogues

The GnRH receptor plays a central role in the regulation of mammalian reproduction (Cheng and Leung, 2005; Millar et al., 2004). Its ligand, GnRH I, is produced by proteolytic cleavage of a precursor polypeptide in the hypothalamus and is released in a pulsatile manner into the hypophyseal portal blood stream. This facilitates transport of the decapeptide to GnRH receptors expressed on gonadotrope cells in the anterior pituitary, initiating GnRH receptor activation (Cheng and Leung, 2005; Millar et al., 2004). GnRH receptor activation mediates the biosynthesis and secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH) which subsequently regulate steroidogenesis and gametogenesis at the gonads (Cheng and Leung, 2005; Millar et al., 2004).

Consistent with this role, mutations of the human GnRH receptor are associated with hypogonadotrophic hypogonadism (HH), a disease characterised by absent or incomplete pubertal development and infertility (Karges et al., 2003). In the case of partial loss-of-function mutations, administration of high pulsatile doses of exogenous GnRH analogues can be used to alleviate this condition (Karges et al., 2003). GnRH agonists and antagonists are also used in the treatment of disorders initiated and/or exacerbated by sex steroids, including precocious puberty, endometriosis, fibroids and sex steroid-dependent cancers of the breast, ovary, prostate and endometrium (Casper, 1991; Maria Comaru-Schally and Schally, 1997). The therapeutic effect of GnRH analogues on these sex steroid-dependent disorders is the indirect result of downregulation (agonists) or competitive inhibition (antagonists) of the GnRH receptor and its signalling, which thereby inhibits the downstream release of sex steroids from the gonads (Casper, 1991; Herbst, 2003).

In addition to GnRH I, there is a second endogenous ligand for the GnRH receptor in humans, GnRH II. GnRH I is the decapeptide, $pGlu^{1}$-His^{2}-Trp^{3}-Ser^{4}-Tyr^{5}-Gly^{6}-Leu^{7}-Arg^{8}-Pro^{9}-Gly^{10}.NH_2 whereas GnRH II differs by three amino acids and has the substitutions His^{5}, Trp^{7} and Tyr^{8}. Interestingly, GnRH II exhibits 100% conservation within species from bony fish to man suggesting that this peptide serves an important,

but as yet elusive, function (Pawson et al., 2003). In some mammalian species, a second receptor, the type II GnRH receptor, is proposed to mediate the signalling of this ligand (Millar, 2003). However, in humans, a frame-shift mutation results in the generation of a premature stop codon in the putative type II receptor transcript, preventing expression of a full length type II receptor (Faurholm et al., 2001). Nevertheless, the human type I receptor binds both GnRH I and GnRH II with high affinity. Thus the type I receptor is proposed to mediate the physiological effects of both ligands in humans (Millar, 2003; Pawson and McNeilly, 2005).

GnRH I and GnRH II exhibit distinct and overlapping expression profiles in diverse tissues (Cheng and Leung, 2005; Hapgood et al., 2005). As discussed, GnRH I is essential in the control of the hypothalamic-pituitary-gonadal axis. In contrast, GnRH II is suggested to play a role in the regulation of sexual behaviour in some mammalian species (Barnett et al., 2006; Kauffman and Rissman, 2004; Kauffman et al., 2005). Nevertheless, GnRHs have been implicated in the regulation of a number of other functions within the neuronal, immune and reproductive systems in both physiological and pathophysiological settings (summarised in Table 1.1). For example, GnRHs are suggested to play a role in immune cell development and neuronal migration, in addition to effects on other aspects of reproduction, such as enhancing fertilisation and implantation. In the pathophysiological context, GnRH analogues have been shown to directly inhibit the proliferation of reproductive cancers, independently of the sex steroid dependence of the cancer. Many of these above-mentioned functions have been suggested by *ex vivo* data following administration of exogenous GnRHs and the physiological relevance and differential effects of the two peptides require further investigation.

The functional diversity and widespread expression of the GnRH receptor and its ligands highlight some important features of this receptor. Firstly, this single receptor, not only binds to, but is also required to transduce signalling to distinct pathways activated by the two structurally distinct ligands, GnRH I and GnRH II. Secondly, the pharmacological and signalling profile of the receptor differs in extrapituitary tissues compared with the

pituitary (Table 1.1 and the references therein). An understanding of the molecular functioning of the GnRH receptor is paramount to address the mechanism whereby this receptor achieves this intricate feat. Here, I review the current understanding of GnRH receptor ligand binding, receptor activation and downstream signalling within the context of a larger group of proteins, the G protein-coupled receptor (GPCR) superfamily.

Table 1.1. Established and putative roles of the GnRH receptor in mammals

Site of expression and action	Established/putative physiological Role	Signalling involved *	GnRH I /GnRH II	Reference(s)
Brain (hypothalamic-pituitary axis)	Control of LH and FSH biosynthesis and release	$G_{q/11}$, G_s, PKA, PKC, MAPK activation and calcium release	GnRH I	(Cheng and Leung, 2005; Pawson and McNeilly, 2005; Rispoli and Nett, 2005)
Ovary	Effects on steroidogenesis, luteinisation, luteolysis and atresia	Induction of apoptosis and upregulated expression of plasminogen activator, MMP-2, membrane type I MMP and prostaglandin endoperoxide synthase II	GnRH I and GnRH II	(Kang et al., 2003)
Embryo and endometrium (decidual stromal and cytotrophoblast cells)	Implantation	Effects on uPA, PAI-1, MMP-2, MMP-9 and TIMP-1 expression	GnRH I and GnRH II (Differential effects)	(Cheng and Leung, 2005; Chou et al., 2003a; Chou et al., 2003b; Chou et al., 2003c; Chou et al., 2002)
Placenta	Stimulation of hCG release/ implantation	NIS	Various GnRH analogues (I and II)	(Rama and Rao, 2001)
Sperm	Enhanced sperm-oocyte interactions/fertilisation	NIS	GnRH I	(Morales, 1998)
Brain	Regulation of sexual behaviour	NIS	GnRH II (not GnRH I)	(Barnett et al., 2006; Kauffman and Rissman, 2004; Kauffman et al., 2005)
Immune cells (B and T cells)	Immune system development (modulation of proliferation and maturation)	NIS	GnRH I	(Tanriverdi et al., 2003)
Immune cells	Stimulation of immune response	Increased IL2R and IFN-γ expression	GnRH I	(Tanriverdi et al., 2003)
Immune cells (T cells)	Adhesion, chemotaxis and homing of T cells	Upregulated expression of the laminin receptor, adhesion to laminin and chemotaxis to SDF-1α	GnRH I and GnRH II	(Chen et al., 2002)
Brain (neuronal cells)	Migration and differentiation	G_i activation and actin remodelling (migration), decreased nestin expression (differentiation)	GnRH I; GnRH I analogue (Buserelin)	(Navratil et al., 2007; Romanelli et al., 2004; Tobet and Schwarting, 2006)

Site of expression and action	**Established/putative physiological Role**	**Signalling involved ***	**GnRH I /GnRH II**	**Reference(s)**
Digestive tract	Inhibition of gastric acid secretion	NIS	GnRH I analogue (Alarelin)	(Chen et al., 2005; Huang et al., 2001)
Melanoma cells	Inhibition of proliferation	NIS	GnRH I analogue (Zoladex)	(Moretti et al., 2002)
Reproductive and cancerous tissues of the breast, ovary, prostate, endometrium and uterus	Inhibition of proliferation and induction of apoptosis ‡	G_i, PTP and SAPK activation. Inhibition of RTK signalling (EGF and IGF-I receptors). Various gene transcription profiles altered (eg. *c-fos*). Inhibition of the PI3K-Akt pathway. †	GnRH I and GnRH II (GnRH II is more potent than GnRH I)	(Grundker and Emons, 2003; Kraus et al., 2006; Limonta et al., 2003)

NIS- no information supplied specific for signalling.
* key, rather than comprehensive, signalling represented here.
† precise signalling here is cell-context dependent.
‡ Under some circumstances proliferative effects have been observed (Grundker and Emons, 2003)

1.2 Introduction to GPCRs and G proteins

The GnRH receptor is a member of the GPCR superfamily. GPCRs are cell surface proteins that recognise a vast array of extracellular signals ranging from photons, ions, nucleotides, lipids and biogenic amines, to peptides and larger proteins (Schlyer and Horuk, 2006). They thus regulate a multitude of physiological functions and are important therapeutic intervention points for numerous illnesses. Indeed, over 30% of all current drugs in clinical use target GPCRs (Jacoby et al., 2006; Schlyer and Horuk, 2006). In this section, an overview of GPCR structure, signalling and trafficking is provided.

1.2.1 Outline of GPCR structure, classification and numbering system

Despite the ability of GPCRs to bind structurally diverse ligands, these proteins are thought to share a common structural architecture. This conserved topology consists of seven transmembrane (7TM) domains linked by three extracellular (ECL) and three intracellular (ICL) loops. Furthermore, an extracellular N-terminal and an intracellular C-terminal domain are present (Fig.1.1).

Analysis of amino acid sequence homology and conserved structural characteristics has allowed classification of GPCRs into 5 main families, the rhodopsin, glutamate, adhesion, frizzled/taste and secretin families (Fredriksson et al., 2003). The rhodopsin family of GPCRs is the largest and the GnRH receptor falls within this category. The conserved structural characteristics of this family are discussed in more detail in section 1.3.

Based on the highly conserved residues within this family, a numbering system for identification of a specific residue within the GPCR structure has been devised. This system, the Ballesteros and Weinstein numbering system, assigns the most highly conserved residue in each TM domain the arbitrary number of 50, in addition to the TM number (Ballesteros and Weinstein, 1995). For example, the most conserved residue in TM6 is a proline residue and is referred to as $Pro^{6.50}$. The positions of other amino acids within the TM are numbered accordingly allowing the reader to identify each residue

relative to this pivotal residue within the TM domain. This numbering system will be used in this review.

1.2.2 GPCR synthesis and trafficking

The predominant pathway for GPCR synthesis and trafficking in the cell involves the following cycle (Ferguson, 2001; Marchese et al., 2003; Moore et al., 2007; Reiter and Lefkowitz, 2006; Tan et al., 2004), which is summarised in Fig.1.2. GPCRs are synthesised, folded and assembled in the endoplasmic reticulum (ER) of the cell (Duvernay et al., 2005). Thereafter, they undergo additional post-translational modifications (such as glycosylation) as they migrate from the ER, through the golgi, to the plasma membrane, where the mature GPCR is finally delivered (Duvernay et al., 2005). There are a number of factors that modulate the GPCR export process. These may include receptor dimerisation and interactions with other proteins, including ER chaperones and accessory proteins, which assist in receptor folding or alter receptor conformation thereby aiding or altering receptor trafficking to the plasma membrane (Duvernay et al., 2005; Tan et al., 2004). Defects in this pathway, resulting from GPCR mutations which prevent their correct folding and export from the ER, cause a number of diseases, such as retinitis pigmentosa, diabetes insipidus and HH (Tan et al., 2004; Ulloa-Aguirre et al., 2004). In the case of GnRH receptor mutations causing HH, cell-permeable non-peptide antagonists (such as IN3) have been shown to rescue expression by acting as a pharmacological chaperone to assist the folding, assembly and routing of the mutant receptor (Ulloa-Aguirre et al., 2004). Once rescued, many of these receptors are able to bind GnRH analogues and evoke cell signalling at levels comparable with the wildtype receptor suggesting that the major defect of these mutations results from impaired trafficking of the receptor to the membrane (Ulloa-Aguirre et al., 2004).

At the plasma membrane, the GPCR is correctly positioned for interaction with its ligand at the extracellular surface as well as with GTP-binding (G) proteins and additional interacting proteins at the intracellular surface. Agonist interaction with the receptor induces a conformational change in receptor structure which facilitates activation of G proteins (Gether, 2000). The active conformation of the receptor also

facilitates the sequential binding of G protein-coupled receptor kinases (GRKs) and β-arrestins which initiate rapid desensitisation of GPCRs (Moore et al., 2007; Reiter and Lefkowitz, 2006). GRKs bind to the receptor first and phosphorylate serine and threonine residues within ICL3 and the C-terminal tail of the receptor (Moore et al., 2007; Reiter and Lefkowitz, 2006). β-arrestins are subsequently recruited and bind to the phosphorylated GPCR sterically uncoupling the receptor from G protein interaction and activation (Moore et al., 2007; Reiter and Lefkowitz, 2006). Arrestin binding also facilitates interaction of the receptor with the internalisation machinery of the cell which enables internalisation of the GPCR via clathrin-coated pits (CCPs) and can initiate a second wave of GPCR-mediated signalling (Marchese et al., 2003; Moore et al., 2007; Reiter and Lefkowitz, 2006; von Zastrow, 2003). Internalised GPCRs are subsequently sorted via a recycling pathway back to the plasma membrane or trafficked to lysosomes where the receptor is degraded (Marchese et al., 2003; Moore et al., 2007; von Zastrow, 2003).

1.2.3 Overview of GPCR signalling

As their name suggests, a central element of GPCR signalling involves activation of heterotrimeric G proteins, which consist of a heterotrimeric complex of Gα, Gβ and Gγ subunits (Cabrera-Vera et al., 2003; McCudden et al., 2005). Gα and Gγ have lipid modifications that anchor the heterotrimer to the plasma membrane facilitating their interactions with GPCRs (Cabrera-Vera et al., 2003; Wedegaertner et al., 1995). Receptor-mediated G protein activation involves the following sequence of events (summarised in Fig.1.3). In the inactive state, the Gα subunit of the heterotrimeric complex is bound to a GDP molecule. Upon agonist activation, GPCRs undergo conformational changes that, in turn, induce conformational alterations in the Gα subunit, promoting the release of GDP and its subsequent association with GTP (Birnbaumer, 2007; Cabrera-Vera et al., 2003; McCudden et al., 2005). In the traditional model of G protein activation, the Gα subunit dissociates from Gβγ, freeing both functional units for regulation of various intracellular effectors (McCudden et al., 2005; Robishaw and Berlot, 2004). However, recent evidence suggests that dissociation is not

absolutely required for activation of the G protein. Thus a second model (the "clamshell" model) exists where G protein activation involves rearrangement, rather than dissociation, of the G protein subunits facilitating exposure of domains necessary for effector activation (Bunemann et al., 2003; Robishaw and Berlot, 2004). G protein inactivation results from the intrinsic GTPase activity of the Gα subunit, which promotes the hydrolysis of GTP to GDP. This facilitates the termination of Gα and Gβγ signalling, either by their reassociation or by conformational changes that obscure effector interaction sites on the heterotrimeric protein (Cabrera-Vera et al., 2003; Robishaw and Berlot, 2004).

In addition to its intrinsic GTPase activity, G protein signalling is regulated by a number of factors (Birnbaumer, 2007). Firstly, interaction with effectors enhances the GTPase activity of the G protein. Furthermore, a dedicated family of proteins, the regulators of G protein signalling (RGS) proteins, have been identified. These proteins have been shown to augment the kinetics of activation and deactivation of G proteins (Birnbaumer, 2007; McCudden et al., 2005). Finally, covalent modifications of G proteins (including acylation or phosphorylation) alter the signalling capacity of G proteins (Chen and Manning, 2001).

Currently, 16 different Gα, 5 Gβ and 12 Gγ genes have been identified in humans (Cabrera-Vera et al., 2003; McCudden et al., 2005). Furthermore, G proteins can be divided into four major classes based on the sequence homology of the Gα subunit, namely G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ (Cabrera-Vera et al., 2003; McCudden et al., 2005). The Gα and Gβγ functional signalling units interact with a number of different intracellular effectors (summarised in Table 1.2 and 1.3 respectively) (Birnbaumer, 2007; Cabrera-Vera et al., 2003).

GPCRs and G proteins are not exclusive partners. G proteins can be activated by other non-GPCR proteins. Emerging evidence suggests that another family of receptors, the receptor tyrosine kinases (RTKs), may recruit and activate G proteins (Kreuzer et al.,

2004; Shan et al., 2006b). Furthermore, non-receptor activators of G protein signalling/AGS proteins can activate G proteins independently of receptors (Cabrera-Vera et al., 2003; Cismowski, 2006; McCudden et al., 2005). Conversely, GPCRs do not only interact with and activate G proteins. Emerging evidence has revealed that GPCRs can activate a number of heterotrimeric G protein-independent signalling pathways (Brady and Limbird, 2002; Hall and Lefkowitz, 2002). For example, GPCRs have been shown to regulate monomeric G proteins (Bhattacharya et al., 2004). GPCRs also enter protein tyrosine phosphorylation cascades independently of heterotrimeric G proteins by direct interaction with Janus kinases (JAKs) (Ali et al., 2000), src family kinases (Cao et al., 2000) and RTKs (Pyne et al., 2003). Additionally, GPCR binding of GRKs, β-arrestins and several other scaffolding proteins can initiate downstream signalling (Hall and Lefkowitz, 2002). Thus these heterotrimeric G protein-independent signalling events increase the diverse signalling capacity of GPCRs.

1.2.4 Summary

GPCRs share a common overall structure, pathways of trafficking in the cell and mechanisms of signalling. In the following sections, our current understanding regarding GnRH receptor structure and signalling will be discussed. However, where necessary, information from pioneering studies with other rhodopsin family GPCRs, such as the prototypical rhodopsin and β_2-adrenergic receptors, will be used to understand and predict the probable mechanism of GnRH receptor molecular functioning.

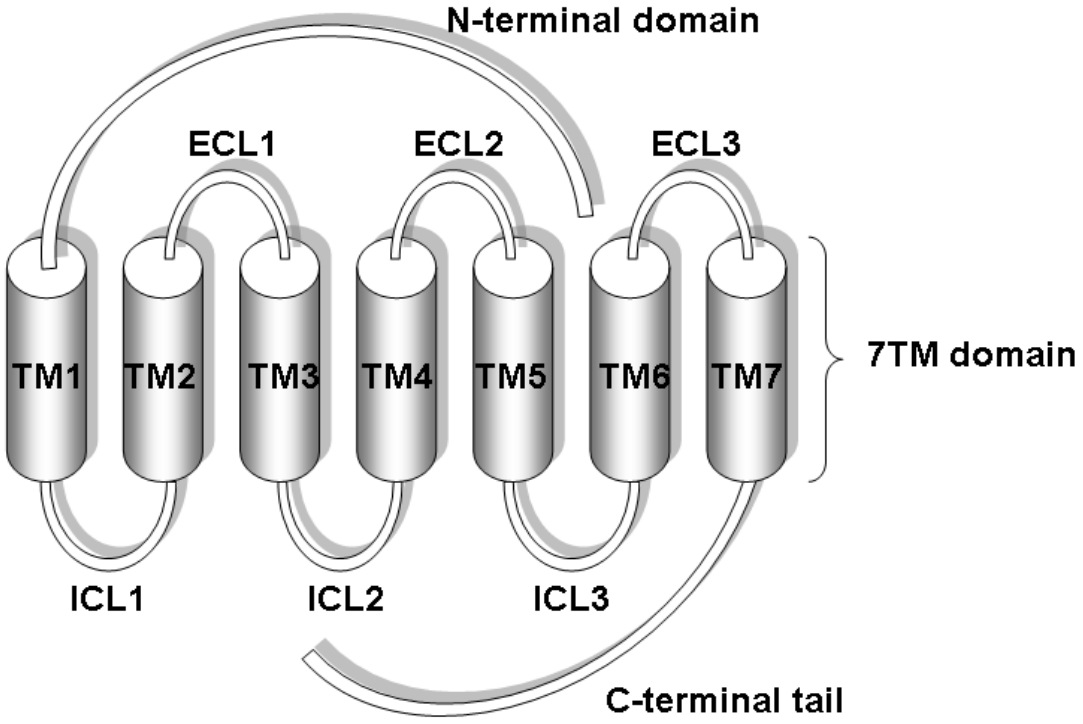


Figure 1.1. Overall topology of the GPCR superfamily. Members of the GPCR superfamily have a conserved structure consisting of seven transmembrane (7TM)-spanning regions linked by three extracellular and three intracellular loops (ECLs and ICLs respectively) and an N- and C-terminal domain.

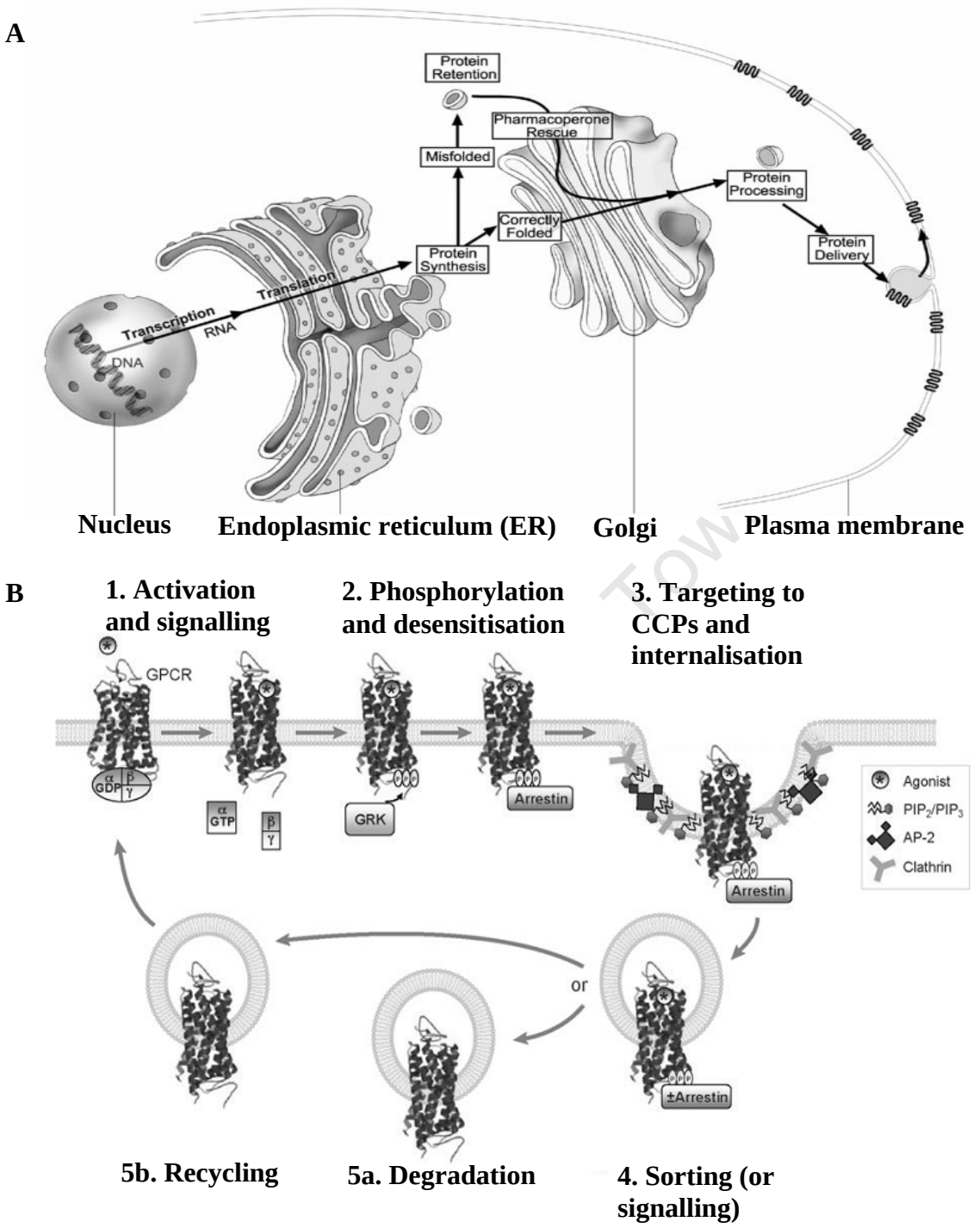


Figure 1.2. GPCR biosynthesis (A) and trafficking (B). A, GPCRs are synthesised, folded and assembled in the ER. They undergo post-translational modifications while migrating through the ER and the golgi and the resulting mature GPCR is delivered to the plasma membrane. Incorrectly folded GPCRs are retained in the ER, but cell-permeable small molecule antagonists (pharmacoperones) can be used to rescue expression by serving as a template to enable correct folding and routing of the receptor. Figure adapted from Conn et al. (Conn et al., 2007). B, Agonist-induced receptor activation facilitates G protein activation and signalling (1). The receptor is then phosphorylated by GRKs creating docking sites for arrestins (2). Arrestin binding enables receptor internalisation by clathrin-coated pits (CCPs) and can initiate a second wave of signalling (3-4). Internalised GPCRs are sorted for degradation or recycling to the plasma membrane (4-5). Figure adapted from Moore et al. (Moore et al., 2007).

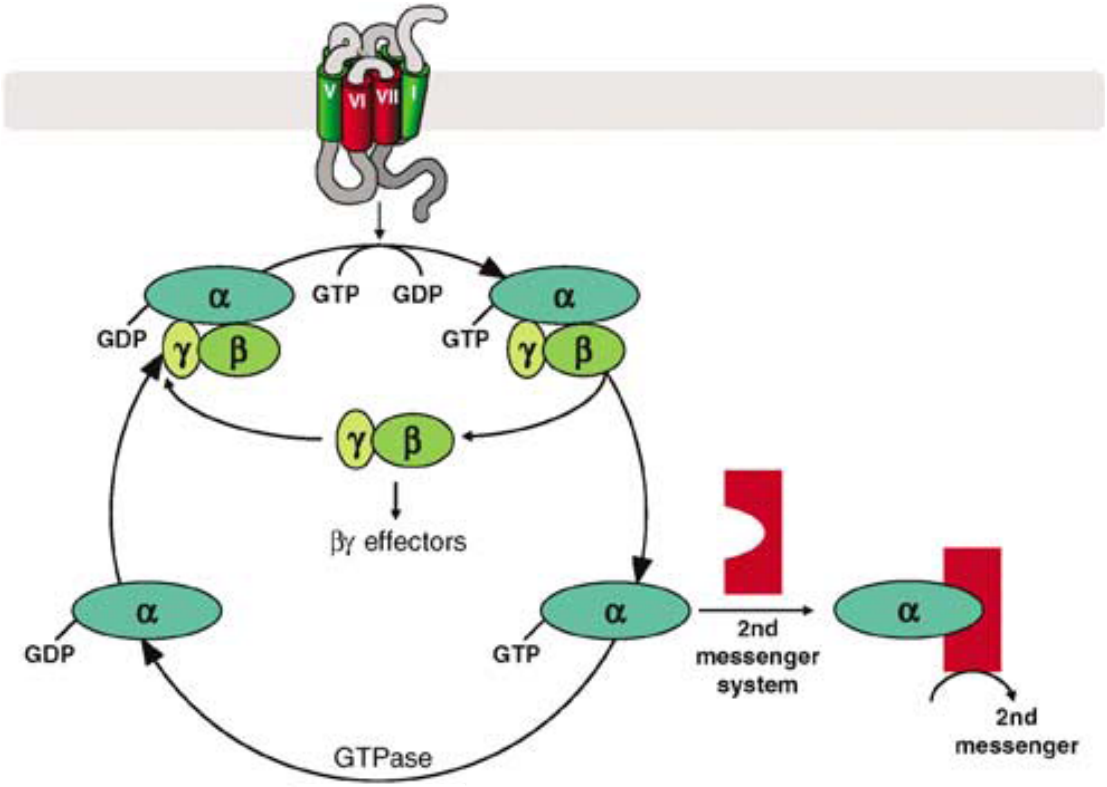


Figure 1.3. The G protein activation cycle. In the inactive state, the Gα subunit of the G protein is bound to a GDP molecule and is associated with the Gβγ subunits. Agonist-induced GPCR activation or constitutive GPCR activity induces a conformational change in the G protein which results in the release of the GDP molecule and the uptake of GTP. This facilitates dissociation of the Gα subunit from Gβγ enabling interaction with and activation of their respective effector molecules. The intrinsic GTPase activity of the Gα subunit facilitates GTP hydrolysis to GDP, inactivation of the G protein and thus reassociation of the heterotrimeric G protein complex. Recent evidence suggests that G protein activation may not necessarily require dissociation of the Gα and Gβγ subunits, but may instead involve rearrangement of the G protein complex (see text for details). Figure taken from Milligan et al. (Milligan and Kostenis, 2006).

Table 1.2. Effectors of the Gα subunits of the heterotrimeric G proteins.

G protein family	Subtype	Effector
G_s	$G\alpha_s$†	↑AC
		↑GTPase of tubulin
		↑src
	$G\alpha_{olf}$	↑AC
G_i	$G\alpha_i$ ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$)	↓AC
	$G\alpha_{i1}$	↑PI3 kinase γ
	$G\alpha_{i2}$	↑K^+ channels
	$G\alpha_i$	↑src
	$G\alpha_o$†	↓AC
		PKA sequestration in extranuclear compartment
	$G\alpha_o$, $G\alpha_{i2}$	↓Ca^{2+} channels
	$G\alpha_{i/o}$	↑Rap1 GAP degradation
	$G\alpha_t$ ($G\alpha_{t1}$, $G\alpha_{t2}$)	↑cGMP-PDE
	$G\alpha_i$, $G\alpha_o$, $G\alpha_z$	Bind GRIN 1 and 2
	$G\alpha_g$	Unknown
G_q	$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, $G\alpha_{16}$	↑ PLCβs
	$G\alpha_q$	↑ Btk
G_{12}	$G\alpha_{12}$	↑Tec kinase tyrosine-phosphorylated LARG
		↑Btk
		↑ras-GAP1
	$G\alpha_{13}$	↑p115-RhoGEF
		↑PDZ-RhoGEF
		↑LARG

Adapted from information in previous reviews (Albert and Robillard, 2002; Birnbaumer, 2007; Cabrera-Vera et al., 2003). † $G\alpha_s$ and $G\alpha_o$ each have two splice variants, $G\alpha_{s(S)}$ and $G\alpha_{s(L)}$ and $G\alpha_{oA}$ and $G\alpha_{oB}$ respectively. Abbreviations are in text and/or Abbreviations section. ↑= stimulate; ↓ = inhibit.

Table 1.3. Regulation of effectors by the Gβγ dimers of heterotrimeric G proteins

Effector regulation
↑PLCβs (not PLCβ4)
↓AC I
↑AC II, IV and VII
↑K^+ channels (including GIRK1, 2, 4)
↓Ca^{2+} channels
Binds and activates GRKs
↑PI3K
↑Btk (indirect action by facilitating interaction with and activation by Gα subunits)
↑Tsk tyrosine kinase
↑PKD
↓Calmodulin kinase
↑ Tubulin GTPase activity
↓Dynamin I GTPase activity
Gβγ acts as a scaffold for Shc and Shc MAPK complex
Raf-1 protein kinase sequesters Gβγ
↑Ras exchange factor
Gβγ sequesters KSR-1
↓transcription of the Glucocorticoid receptor in the nucleus

Table is adapted from previous reviews (Birnbaumer, 2007; Cabrera-Vera et al., 2003).
↑ = stimulate; ↓ = inhibit. Abbreviations in text and/or Abbreviations section.

1.3 GnRH receptor structure

Knowledge of a GPCR's structure is useful in understanding the mechanisms of ligand binding and receptor activation. This information can be used to refine computational models of the receptor, allowing the user to dock ligands to experimentally-identified ligand binding sites and thereby predict novel receptor sites where additional ligand-receptor interactions may occur. Computational models are also useful in ligand design and can facilitate identification of lead compounds for high throughput drug screening assays (Fanelli and De Benedetti, 2005).

GPCRs are thought to share a similar three-dimensional structure consisting of a bundle of 7 transmembrane spanning-alpha helices orientated roughly perpendicular to the plasma membrane (Filipek et al., 2003b; Yeagle and Albert, 2007), connected by 3 ICLs and 3 ECLs and with an N- and C-terminal domain. The proposal that GPCRs share a common structural architecture, particularly in the TM domains, is based on the conservation of key structurally relevant sequences within the rhodopsin family GPCRs (despite the low overall sequence homology) and the common requirement of these receptors to interact with and activate a small subset of highly homologous G proteins (Baldwin, 1993; Ballesteros et al., 2001b; Mirzadegan et al., 2003). This proposal has facilitated the creation of successful computational models of GPCRs, as discussed below.

Previously, the relative orientation of residues within the 7TM bundle of GPCRs was determined by sequence analysis of the size, hydrophobicity and global sequence conservation of residues within the helices of GPCRs (Baldwin, 1993; Ballesteros and Weinstein, 1995). Specifically, residues that are hydrophobic, but not restricted in size and exhibit low sequence conservation were predicted to face outwards towards the lipid bilayer (Baldwin, 1993; Ballesteros and Weinstein, 1995). Conversely, conserved and/or hydrophilic residues, which exhibit size restrictions, were predicted to face into the TM bundle and participate in interhelical interactions (Baldwin, 1993; Ballesteros and Weinstein, 1995). Models of GPCR structure built *ab initio* using these principles, together with data from low resolution electron density maps and biochemical and

biophysical experiments, were essentially consistent with the crystal structure of rhodopsin that emerged subsequently (Ballesteros et al., 2001b; Fanelli and De Benedetti, 2005; Yeagle and Albert, 2007). The elucidation of the crystal structure of rhodopsin provided the first direct structural information regarding the inactive state of a GPCR (Li et al., 2004; Okada et al., 2004; Palczewski et al., 2000). The fact that it was consistent with the previous predictions supported the premise upon which these data were based; specifically that rhodopsin family GPCRs have a similar overall structure. Thus the high resolution structural data obtained from the crystal structure of the rhodopsin provides the best information to date to accurately model the 7TM domains of other GPCRs in the family (Fanelli and De Benedetti, 2005).

Due to the difficulty of crystallisation, molecular models of the GnRH receptor have been built by homology modelling based on the crystal structure of rhodopsin (Betz et al., 2006; Lu et al., 2007; Lu et al., 2005; Soderhall et al., 2005). These models provide useful information regarding proposed inter- and intramolecular interactions, which can then be tested by mutagenesis. The mutagenesis results can then be used to refine the model. In this section, information regarding the crystal structure of rhodopsin is presented and compared with the primary sequence and experimental data specific to the GnRH receptor. An annotated sequence alignment of rhodopsin and the GnRH receptor is presented in Figure 1.4.

1.3.1 The 7TM region

The crystal structure of rhodopsin reveals that the 7TM helices are arranged in an anti-clockwise direction, when viewed from the extracellular surface (see Fig. 1.5). While they are orientated roughly perpendicular to the membrane, many of the helices are tilted, appearing to bend or lean over the neighbouring helix. A number of residues, such as prolines, disrupt the regularity of the helices, inducing kinks within the TM segments (see Fig 1.5). These structural kinks and irregularities change the trajectory of the TM helices, creating a flask-like-shaped structure from a protein that would otherwise have resembled a barrel. The advantages of this structure are inherent in its function as it generates three functional domains within the receptor, an extracellular region for ligand

binding, an intracellular region for interaction with intracellular effectors and a mid-region which provides a means of transduction between the two (Madabushi et al., 2004). Furthermore, this conformation facilitates a number of important interhelical interactions. For example, TM3, while not significantly kinked, is tilted from the membrane normal by ~30^{o}. It thus changes its position within the 7TM bundle by traversing from its inception point between TM2 and TM4 at the extracellular surface of the molecule to its termination point at the intracellular surface in close proximity to TM5. This facilitates intramolecular interactions between TM3 and five other helices (TM2, TM4, TM5, TM6 and TM7), suggesting an important directive role for this helix in receptor activation (Filipek et al., 2003a).

Sequence alignments of the TM domains of rhodopsin and the GnRH receptor (and indeed other rhodopsin family GPCRs) reveal relatively low homology (see Fig.1.4). However, it is suggested that rhodopsin family GPCRs can, in fact, adopt a similar structure to rhodopsin, despite this low overall sequence identity, by a mechanism known as structural mimicry (Ballesteros et al., 2001b). The basis for this phenomenon is that structural features induced by a particular residue(s) can be recreated by another set of structurally distinct amino acids allowing the receptor to diverge sufficiently to achieve ligand and signalling selectivity while retaining a conserved and efficient functionally-relevant fold for G protein activation. An example of this can be observed in rhodopsin where a kink in TM2, usually associated with the presence of a proline residue, is induced by a set of two glycine and two threonine residues (Palczewski et al., 2000). Analysis of the sequence alignment of rhodopsin and the GnRH receptor in TM2 reveals a proline residue in the GnRH receptor in an analogous position to this Gly-Gly-X-Thr-Thr motif in rhodopsin, thus indicating that this proline may induce a similar kink in TM2 of the GnRH receptor. The GnRH receptor also possesses the highly conserved proline residues within its sequence, notably Pro$^{5.50}$, Pro$^{6.50}$ and Pro$^{7.50}$.

Despite their relatively low sequence homology, the TM domains are the most highly conserved region amongst GPCRs, unlike the extra- and intracellular domains which are more divergent. A few key residues have been identified that exhibit 80-100% sequence

homology (Mirzadegan et al., 2003). These are Asn$^{1.50}$; Leu$^{2.46}$; Asp/Asn$^{2.50}$; Cys$^{3.25}$; Glu/Asp$^{3.49}$; Arg$^{3.50}$; Trp$^{4.50}$; Phe$^{6.44}$; Trp/Phe$^{6.48}$; Pro$^{6.50}$, Pro$^{7.50}$ and Tyr$^{7.53}$ (Fanelli and De Benedetti, 2005; Mirzadegan et al., 2003). Conceivably, some of these residues participate in key structural motifs that direct the overall receptor fold, such as the proline residues (which were discussed above) as well as Cys$^{3.25}$ which forms part of a highly conserved disulfide bridge (see section 1.3.2). The other residues have been identified as playing a role in creation of important intramolecular and interhelical interactions, identified by mutagenesis studies in many rhodopsin family GPCRs as well as in the crystal structure of rhodopsin (Filipek et al., 2003a; Lu et al., 2002). The presence of these highly conserved residues, which direct receptor structure by facilitating key interhelical interactions, provides further support for the similar overall configuration of rhodopsin family GPCRs. Notably, Glu/Asp$^{3.49}$ and Arg$^{3.50}$ form part of the E/DRY motif and the Pro$^{7.50}$ and Tyr$^{7.53}$ residues form part of the D/NPxxY motif. These highly conserved motifs are hallmarks of the rhodopsin family GPCRs and play key roles in receptor activation and will be revisited in more detail in section 1.4.

In the crystal structure of rhodopsin, one set of interactions observed in this inactive state involves a hydrogen bonding network between residues Asn$^{1.50}$, Asp$^{2.50}$ and Asn$^{7.49}$. The Asp$^{2.50}$ and Asn$^{7.49}$ interact via a water molecule (Li et al., 2004; Okada et al., 2004; Palczewski, 2006). Interestingly, this interaction was predicted prior to the crystal structure, following the observation that these highly conserved residues are reciprocally mutated in the GnRH receptor (Zhou et al., 1994). In other words, in the GnRH receptor, position 2.50 is an Asn while position 7.49 is occupied by an Asp. Further experimentation involving site-directed mutagenesis of the GnRH receptor revealed that when Asn$^{2.50}$ was mutated to an Asp in the GnRH receptor, receptor expression was abolished suggesting disruption of an important stabilising intramolecular interaction (Flanagan et al., 1999; Zhou et al., 1994). Consistent with this, when both Asn$^{2.50}$ and Asp$^{7.49}$ were reciprocally mutated recreating the configuration in other GPCRs, GnRH receptor expression and high affinity agonist and antagonist binding were recovered (Flanagan et al., 1999; Zhou et al., 1994). This provides support for the proposal that an analogous network of interactions directs a similar overall fold of the GnRH receptor

compared with rhodopsin. This has enabled successful implementation of homology modelling of the 7TM domains of the GnRH receptor, based on the crystal structure of rhodopsin (Betz et al., 2006; Lu et al., 2007; Lu et al., 2005; Soderhall et al., 2005).

1.3.2 The extracellular domains

Sequence alignment of rhodopsin and the GnRH receptor's extracellular domain (consisting of the N-terminus and ECL1, ECL2 and ECL3) reveal that they are highly divergent (Fig.1.4). Indeed, this domain is the most dissimilar region within rhodopsin family GPCRs, reflecting the requirement of these proteins to interact with a range of structurally distinct ligands (Mirzadegan et al., 2003). In rhodopsin, the extracellular domain forms a compact structure (Li et al., 2004; Okada et al., 2004; Palczewski et al., 2000). ECL1 and ECL3 form short loops and the ECL2 and N-terminal domains form a layered roof of two β-hairpins (composed of 4 β-sheets) arranged over the centre of the 7TM helical bundle (see Fig.1.5). ECL2 forms the innermost β-hairpin and rests within the transmembrane helices. It has often been referred to as a plug/lid and has been suggested to play a role in preventing rapid dissociation of rhodopsin's ligand (Filipek et al., 2003a; Palczewski, 2006; Sakmar, 2002). Experiments by Sakmar and colleagues suggest a major role of ECL2 in rhodopsin in the regulation of receptor activation (Sakmar, 2002). Consistent with the latter role, site-directed mutagenesis of two residues in ECL2 of the GnRH receptor were able to convert an antagonist to an agonist, as measured by inositol phosphate (IP) signalling, suggesting that this region may play a similar role in the GnRH receptor (Ott et al., 2002). In rhodopsin, the β-hairpin of ECL2 is stabilised by a disulfide bridge linking ECL2 to the top of TM3 (Palczewski, 2006; Sakmar, 2002). This disulfide bridge is highly conserved amongst rhodopsin family GPCRs (Mirzadegan et al., 2003) and is also observed in the GnRH receptor (Cook and Eidne, 1997). While retaining cell surface expression, site-directed mutagenesis of the relevant cysteine residues in the GnRH receptor revealed the absolute requirement of this structural motif in ligand binding and receptor activation of this receptor (Cook and Eidne, 1997). However, despite these similarities, it seems unlikely that the extracellular domain of the GnRH receptor is similar to rhodopsin as the deeply plugged position of

ECL2 observed in rhodopsin obscures the GnRH binding pocket. Indeed, computational docking of the GnRH receptor's decapeptide ligands requires a significant displacement of ECL2 and the N-terminal domain.

Other features of the GnRH receptor extracellular domain include a second set of cysteine residues which form a disulfide bridge between the N-terminal domain and ECL2, which is not observed in rhodopsin (Cook and Eidne, 1997). The precise functional role of this bridge is unknown as mutagenesis did not significantly affect cell surface expression, ligand binding or receptor activation (Cook and Eidne, 1997). However, its presence is suggestive of a different conformation of the GnRH receptor's extracellular domain compared with rhodopsin. In addition, the GnRH receptor's N-terminal domain is decorated with glycosylation. The number of glycosylation sites for the GnRH receptor depends on the species and is correlated with increasing receptor expression, but doesn't significantly alter ligand binding affinity or receptor activation (Davidson et al., 1995; Millar et al., 2004). This is consistent with the presence and outward facing arrangement of these carbohydrate moieties in rhodopsin's crystal structure (Li et al., 2004; Okada et al., 2004; Palczewski et al., 2000). While detailed site-directed mutagenesis data and biochemical and biophysical analyses can provide valuable information to model this region of the receptor, only a high resolution structure of the receptor will enable determination of the structure conclusively.

1.3.3 The ligand binding pocket

In the inactive state, the ligand for rhodopsin is the small molecule, 11-*cis*-retinal. It is covalently bound to the receptor at Lys$^{7.43}$ in TM7 by a shiff base linkage, placing it deep within the crevice of the TM bundle (Fig.1.5) (Filipek et al., 2003a). A number of residues in rhodopsin in the 7TM domains and ECL2 of rhodopsin make direct contacts with retinal (Filipek et al., 2003a; Li et al., 2004). Another key contact residue is Glu$^{3.28}$ in TM3, which is suggested to stabilise the inactive conformation of the chromophore by acting as a counterion for the protonated shiff base (Filipek et al., 2003a; Yeagle and Albert, 2007). Furthermore, three highly conserved residues in TM5 and TM6, Phe$^{5.47}$,

$Trp^{6.48}$ and $Tyr^{6.51}$, make important interactions with the β-ionone ring of the 11-*cis*-retinal (Li et al., 2004; Palczewski, 2006; Yeagle and Albert, 2007).

Unlike rhodopsin's ligand, GnRH I and GnRH II decapeptides are much larger, thus preventing their insertion deep within the transmembrane domains and necessitating a different binding mode. The high affinity conformation of these ligands requires the peptide to assume a βII'-type turn involving positions 5-8 of the ligand (Barran et al., 2005; Millar et al., 2004). In this conformation, the peptide resembles a horse-shoe shape where the N- and C-termini are in close proximity and is optimal for ligand-receptor interactions (Millar et al., 2004). This suggested conformation is supported by the recent Nuclear Magnetic Resonance (NMR) structure for GnRH I (deposited in the Protein Data Bank with the pdb code 1YY1).

Due to the absence of crystal structure information for the GnRH receptor, knowledge of ligand-receptor interactions is mainly obtained from site-directed mutagenesis of the GnRH receptor and computational modelling (Millar et al., 2004; Sealfon et al., 1997). Site-directed mutagenesis of the GnRH receptor followed by ligand binding assays to assess ligand binding affinity has enabled identification of potential ligand interaction sites. However, this data must be interpreted with caution, because the mutation of a receptor residue which decreases ligand binding affinity does not necessarily reflect the loss of a direct ligand-receptor interaction site (Sealfon et al., 1997). Mutation of residues in the receptor can indirectly affect ligand binding by disrupting intramolecular interactions that are involved in configuration of the ligand binding pocket (Fromme et al., 2004; Sealfon et al., 1997). This caveat underlines the necessity for verification of potential ligand-receptor interaction sites identified by site-directed mutagenesis of the receptor. Thus if a receptor mutation is identified as decreasing affinity for the ligand, its role in direct ligand interactions should be assessed by concurrent alteration of the ligand. Thus if the ligand and receptor residue interact, substitutions in the ligand that prevent this interaction should result in analogue which has similar affinity at the wildtype and mutant receptors (Millar et al., 2004). The proposed interaction should then

be analysed further by computational modelling to assess the validity of the suggested interaction, in the context of previously identified interactions. While the best method to assess ligand binding affinity involves the use of ligand binding assays, mutations which induce very low affinity can prevent the accurate use of this technique. However, in cases where the mutant receptors have comparable cell surface expression relative to the wildtype receptor, the measurement of second messenger generation, such as IP_3, can be used (Mamputha et al., 2007). In addition, the introduction of conservative mutations may minimise the structural disruption caused by mutation of the specific residue and substitution with smaller amino acids, like alanine, can avoid the introduction of novel intramolecular interactions.

Combining the results of site-directed mutagenesis of the GnRH receptor in competition binding and functional assays with homology modelling of the GnRH receptor based on the crystal structure of rhodopsin (Lu et al., 2007; Stewart et al., 2007), a number of interactions between GnRH I and the human GnRH receptor have been identified (see Fig.1.6A). In the following section, the experimental and computational evidence to support them is described. This is presented in sequential order of the residues in the ligand and their proposed interactions with the receptor. Interactions, where additional supporting experimental evidence is required, are highlighted.

1.3.3.1 pGlu^{1} forms a H-bond with Asn$^{5.39}$

The mutation of Asn$^{5.39}$ to Ala induces a decrease in affinity for GnRH I compared with the wildtype receptor, as was inferred by a decrease in potency (Hoffmann et al., 2000). This decrease was smaller when measured at the Asn$^{5.39}$Gln receptor, which has a conservative mutation at this position (Hoffmann et al., 2000). This suggests that the side chain of Asn$^{5.39}$ is important for GnRH I binding and that this function can be partially recapitulated with a glutamine side chain. The authors used molecular modelling to suggest an interaction of the Asn$^{5.39}$ side chain with the backbone ketone group of His^{2} (Hoffmann et al., 2000). However, subsequent refinements of the GnRH receptor molecular model suggest that this interaction is more likely to involve a H-bond

with $pGlu^1$ (Millar et al., 2004). Experiments with $pGlu^1$-substituted GnRH analogues are required to confirm this proposed interaction.

1.3.3.2 His^2 interacts with $Asp^{2.61}$ and $Lys^{3.32}$

The role of $Lys^{3.32}$ in GnRH I binding and the nature of the interaction was suggested following a series of mutations of $Lys^{3.32}$ to Arg, Gln and Leu (Zhou et al., 1995). These receptor mutations provide an indication of the nature of the ligand-receptor interaction by analysis of their side chain characteristics. The wildtype receptor has a lysine residue which has the capacity to act as a hydrogen bond donor and has a positive charge which facilitates ionic interactions. Mutation to Arg is conservative and thus the residue retains these interaction capabilities. Consistent with this, the $Lys^{3.32}$Arg receptor retained high affinity binding and produced functional responses comparable with the wildtype receptor. However, mutation to Gln, which retains the ability to form H-bonds, but lacks the positive charge, abolished detectable ligand binding and the receptor produced a functional response with a much higher EC_{50} value. This suggests that the charge of Lys is important for high affinity binding. Finally, mutation to Leu, which eliminates both the charge and H-bonding capacity of the residue, resulted in a receptor unable to produce a functional response, suggesting that a charge-strengthened hydrogen bond donor is required at this position in the receptor in order to facilitate high affinity binding of GnRH I. The authors postulate that this interaction may occur with His^2 or Trp^3 of GnRH I, but subsequent experiments with modified ligands to confirm the proposed interactions, were not performed.

The involvement of $Lys^{3.32}$ in ligand binding was elucidated further in a subsequent paper that identified and investigated the role of $Asp^{2.61}$ in the binding of GnRH I. Mutation of $Asp^{2.61}$ to the conserved Glu (which has the same charge, but an increased side chain length) and then to an uncharged residue Asn/Ala/Val resulted in a successive decrease in affinity for GnRH I (Flanagan et al., 2000). These data suggest that both the charge and the position of the Asp side chain are required for high affinity GnRH I binding. In contrast with the wildtype receptor, the $Asp^{2.61}$ mutant receptors exhibited a smaller decrease in affinity for His^2-substituted ligands compared with native GnRH I,

suggesting that $Asp^{2.61}$ interacts with His^{2} of GnRH I (Flanagan et al., 2000). Further analysis of a series of substitutions in position 2 of the ligand, as well as calculation of the fold changes in affinity, indicated that the δ-NH group of His^{2} forms a H-bond with $Asp^{2.61}$. However, this interaction did not explain the loss of affinity of all peptides (irrespective of ligand modifications) at the $Asp^{2.61}$Val receptor compared with the $Asp^{2.61}$Glu receptor (Flanagan et al., 2000). Compared with the Glu mutation, the Val mutation lacks negative charge. This suggests that this charge at this position in the receptor is required in formation of interactions that facilitate creation of the ligand binding pocket or is involved in an interaction with the backbone of the peptide. Computational modelling revealed a potential ionic interaction with $Lys^{3.32}$. This interaction is proposed to facilitate $Lys^{3.32}$ side chain interactions with the ligand (Flanagan et al., 2000). Our current molecular model suggests that the $Lys^{3.32}$ interaction is with His^{2}, but this requires experimental support.

1.3.3.3 Trp^{3} and Ser^{4} interactions

Studies involving mutation of $Trp^{6.48}$ to Ser in the rat GnRH receptor showed a small (~2-3-fold) decrease in affinity for GnRH I compared with the wildtype receptor (Chauvin et al., 2000; Chauvin et al., 2001). The authors suggested that this residue was important in the binding of GnRH I (Chauvin et al., 2000; Chauvin et al., 2001). We have subsequently mutated this residue in the human GnRH receptor. This mutation induced a large decrease in receptor expression (Coetsee et al., 2006). However, following rescue of receptor expression using the small molecule antagonist IN3 (Lu et al., 2005), no decrease in affinity for GnRH I or GnRH II was observed at the $Trp^{6.48}$-mutated receptors compared with the wildtype receptor (Coetsee et al., 2006). This is consistent with a previous observation that $Trp^{6.48}$ was not involved in GnRH I binding (Betz et al., 2006). The position of Trp^{3} in our current molecular model suggests that it may make interactions with ECL2.

The interactions between Ser^{4} and the GnRH receptor have not been experimentally determined and the role of this residue awaits further investigation. The presence of this residue in the mid-region of the peptide and the observation that larger amino acid

substitutions are not well-tolerated suggests that it may play a role or facilitate the configuration of the high affinity ligand conformation (Millar et al., 2004; Sealfon et al., 1997).

1.3.3.4 Tyr^{5} interacts with Tyr$^{6.58}$

Several groups have mutated Tyr$^{6.58}$ and observed large decreases in affinity for GnRH I (Betz et al., 2006; Hovelmann et al., 2002). Molecular modelling revealed that this interaction could be with Tyr^{5} (Hovelmann et al., 2002), but this interaction requires experimental support. In chapter 2, using Tyr$^{6.58}$-mutated receptors and position 5-substituted GnRH I and GnRH II analogues, I investigate the validity and nature of this proposed interaction.

1.3.3.5 Gly^{6} and Leu^{7} interactions

Receptor interactions with Gly^{6} are not well-established. However, the importance of a flexible residue or D-amino acid in this position for high affinity binding suggests that this residue contributes to the βII'-type turn required for ligand-receptor interactions (Barran et al., 2005). Experimentally identified receptor interactions with Leu^{7} are still required.

1.3.3.6 Arg^{8} interacts with Asp$^{7.32}$

Mutation of Asp$^{7.32}$ to Asn decreased affinity of the receptor for GnRH I compared with the wildtype receptor, but had no effect on the affinity for Arg^{8}-substituted ligands (Fromme et al., 2001). This suggests that Asp$^{7.32}$ forms an ionic interaction with Arg^{8} of GnRH I. However, further experimental data revealed this interaction to be more complex. Firstly, the reduction in binding affinity for GnRH I observed at the Asp$^{7.32}$-mutated receptor was much smaller than the loss expected for disruption of an electrostatic interaction (Fromme et al., 2001). Furthermore, conformationally constrained ligands, such as those with D-amino acids in position 6, do not exhibit selectivity for Asp$^{7.32}$ and have similar affinities at the mutant and wildtype receptors (Fromme et al., 2001). This led the authors to suggest that Arg^{8} may participate in a

transient ligand-receptor interaction which facilitates creation of the high-affinity conformation of GnRH I.

1.3.3.7 Pro^{9} and the C-terminal glycinamide form interactions with Arg$^{1.35}$ and Asn$^{2.65}$

The interaction of Arg$^{1.35}$ and Asn$^{2.65}$ (in TM1 and TM2 respectively) with the C-terminal glycinamide of the native GnRH peptide has been suggested using GnRH analogues where the glycinamide is substituted with an ethylamide. In both the Arg$^{1.35}$Ala and Asn$^{2.65}$Ala mutant receptors, the observed decrease in affinity (Arg$^{1.35}$Ala) or potency (Asn$^{2.65}$Ala) compared with the wildtype receptor was smaller for the ethylamide-substituted peptides than for native GnRH I (Davidson et al., 1996; Hoffmann et al., 2000; Stewart et al., 2007). The authors suggest that Arg$^{1.35}$ and Asn$^{2.65}$ interact with the C-terminal glycinamide via H-bonds (Davidson et al., 1996; Hoffmann et al., 2000; Stewart et al., 2007). However, the large decreases in affinity/potency of GnRH I at the two mutant receptors compared with the wildtype receptor are not accountable for by the loss of a single H-bond interaction. Indeed, computational modelling suggests that Arg$^{1.35}$ and Asn$^{2.65}$ form part of a hydrogen bonding network that links TM1, TM2 and TM7 and may thereby contribute to receptor structure and stabilisation of the ligand binding pocket (Stewart et al., 2007). Furthermore, Arg$^{1.35}$ may make an additional H-bond with the carbonyl oxygen of Pro^{9} of the GnRH peptide (Stewart et al., 2007).

1.3.3.8 Binding of GnRH II

The ligand-receptor binding residues for GnRH II are less well-defined (Millar et al., 2004). However, a number of interaction sites are suggested to overlap with the GnRH I binding sites (see Fig.1.6B). These include Asp$^{2.61}$, Asn$^{2.65}$, Lys$^{3.32}$ and Arg$^{1.35}$ (Davidson et al., 1996; Flanagan et al., 2000; Millar et al., 2004; Stewart et al., 2007). Nevertheless, an important observation is that mutation of Asp$^{7.32}$ does not affect the binding of GnRH II (which has a tyrosine in position 8) (Fromme et al., 2001). This suggests that Asp$^{7.32}$ confers selectivity for GnRH I by interaction with Arg^{8}. Thus Tyr^{8} of GnRH II is proposed to make differential interactions with the receptor (Lu et al., 2005). Interestingly, in our molecular model of GnRH II docked to the GnRH receptor, Tyr^{8} of

GnRH II faces away from $Asp^{7.32}$, consistent with the proposal that position 8 of GnRH II makes differential interactions with the receptor (see Fig.1.6B).

1.3.3.9 Conservation of analogous ligand binding sites compared with other GPCRs

Interestingly, a number of residues at the equivalent positions of the GnRH receptor binding sites are also involved in ligand binding in other GPCRs (Millar et al., 2004). An example is $Lys^{3.32}$, which is at the equivalent position of $Asp^{3.32}$ in the monoamine receptors, which is important for interaction with the positively charged bioamine headgroups (Shi and Javitch, 2002) and of $Ala^{3.32}$ in rhodopsin, which is also an interaction site for 11-*cis*-retinal (Millar et al., 2004; Palczewski et al., 2000). This indicates that certain GPCR ligand binding sites have been retained to some extent during evolution, but the amino acids have been changed to mirror the chemical nature of the cognate ligands.

1.3.4 The intracellular domains

The intracellular domains of GPCRs consist of three intracellular loops and a C-terminal tail. Crystallisation of rhodopsin has revealed a high degree of flexibility within these regions (Li et al., 2004; Okada et al., 2004; Palczewski, 2006; Palczewski et al., 2000). This is consistent with other experimental evidence suggesting these regions undergo conformational changes following receptor activation (Yeagle and Albert, 2007) and reflects the requirement of these domains to interact with and activate different classes of G proteins and other signalling molecules (Wong, 2003). In the crystallised inactive state of rhodopsin the intracellular loops form random structures that outline the periphery of the receptor (Li et al., 2004; Okada et al., 2004; Palczewski, 2006; Palczewski et al., 2000). In contrast, the C-terminal tail forms an eighth alpha helix which lies parallel to the membrane.

Considering the high sequence divergence of rhodopsin and the GnRH receptor in the intracellular loops and the interaction of the receptors with different G protein subtypes, it is difficult to predict the degree of structural similarity between the two proteins within these regions (see Fig.1.4). Furthermore, the disorganised nature of the loops in

the crystal structure of rhodopsin questions the utility of such a comparison. Nevertheless, a noteworthy observation is the complete absence of a C-terminal tail in the GnRH receptor. This evolutionary alteration in the GnRH receptor is likely to reflect refinement of the receptor for its own specialised signalling (Pawson et al., 1998). Indeed, it has been hypothesised that, because this modification prevents the rapid desensitisation and internalisation of the GnRH receptor, it assists the prolonged LH surge induced by the GnRH receptor, which is required for ovulation in mammals (Pawson et al., 1998).

1.3.5 Insights from the recent crystallisation of the β_2-adrenergic receptor

Current GnRH receptor molecular models are based on the crystal structure of rhodopsin (Betz et al., 2006; Lu et al., 2007; Lu et al., 2005; Soderhall et al., 2005). However, as discussed above, the differences in ligand binding and signalling mediated by the two receptors make it particularly difficult to model the extracellular and intracellular domains of the GnRH receptor based on rhodopsin and limits the utility of these models. Recently, a crystal structure of a second rhodopsin family GPCR, the β_2-adrenergic receptor (β_2-AR), was obtained (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007). In order to achieve crystals, the structural flexibility of the β_2-AR was reduced by complexing the receptor with an antibody to ICL3 (Rasmussen et al., 2007) or by insertion of T4-lysozyme (T4L) in place of ICL3 (Cherezov et al., 2007; Rosenbaum et al., 2007), in combination with the presence of the high affinity partial inverse agonist, carazolol. The antibody-GPCR complex only allowed resolution of the intracellular region of the receptor, but the β_2-AR-T4L was better resolved. This structure is similar to rhodopsin, but exhibits a few important differences. Firstly, the extracellular segments of the TM domains are angled away from the centre of the TM bundle creating a more open conformation at this region of the receptor compared with rhodopsin (Cherezov et al., 2007). Furthermore, unlike ECL2 of rhodopsin, which forms a β-hairpin and is inserted into the TM bundle, ECL2 of the β_2-AR-T4L forms an unexpected short helical segment and is more exposed to the solvent, a conformation which is proposed to prevent ECL2 from blocking access of the ligand to the binding

site (Cherezov et al., 2007). This open configuration of the extracellular region of the β_2-AR is likely to reflect the requirement of this receptor to interact diffusible ligands, which contrasts with the covalently bound ligand at rhodopsin. This is consistent with the observation, mentioned above, that docking of the GnRH receptor's diffusible peptide ligands to the computational models of the receptor requires significant displacement of ECL2. A second important difference is that TM3 is further away from TM6 in the β_2-AR-T4L structure compared with rhodopsin, preventing an interaction known as the "ionic lock" (see section 1.4.3.4), which is proposed to constrain the receptor in the inactive state. This difference may explain the high basal activity of the β_2-AR, which is not completely reduced in the presence of carazolol. This contrasts with the absence of basal activity observed at both rhodopsin and the GnRH receptor. The differences observed between rhodopsin and the β_2-AR highlight that, while homology modelling is a useful tool for identification of putative ligand-receptor and intramolecular interactions, GPCRs have subtle structural alterations that reflect specialisation of the receptor for the binding of different ligands and differential signalling requirements. Thus predicted interactions proposed from homology models require experimental validation, which can be used to refine and thus improve the models.

1.3.6 Summary

The current predicted structure of the GnRH receptor relies heavily on information gained from the crystallisation of another rhodopsin family GPCR, rhodopsin. These receptors share important highly conserved structural motifs that are likely to direct a similar overall fold for the two receptors (Ballesteros et al., 2001b). These motifs include the highly conserved proline residues and residues of the conserved E/DRY and D/NPxxY motifs (Mirzadegan et al., 2003). However, it is clear that the GnRH receptor has a subtly distinct structure compared with rhodopsin, necessary for its own specialised signalling functions. This is observed by the absence of the C-terminal tail and reciprocal change of the Asp and Asn residues in positions 2.50 and 7.49 in the GnRH receptor (Flanagan et al., 1999; McArdle et al., 1999; Zhou et al., 1994). Thus,

homology models of the GnRH receptor based on the structure of rhodopsin should be subject to refinements which incorporate experimental data specific to the GnRH receptor. Nevertheless, the validity and predictive power of GnRH receptor models, based on the structure of rhodopsin, have been obtained from experimental evidence that supports the proposed interactions extrapolated from the models (Millar et al., 2004). An important underlying implication of the conserved structure of rhodopsin family GPCRs is that they undergo a common mechanism of activation. In the following section, the role of conserved residues and motifs in a proposed global mechanism of activation is discussed.

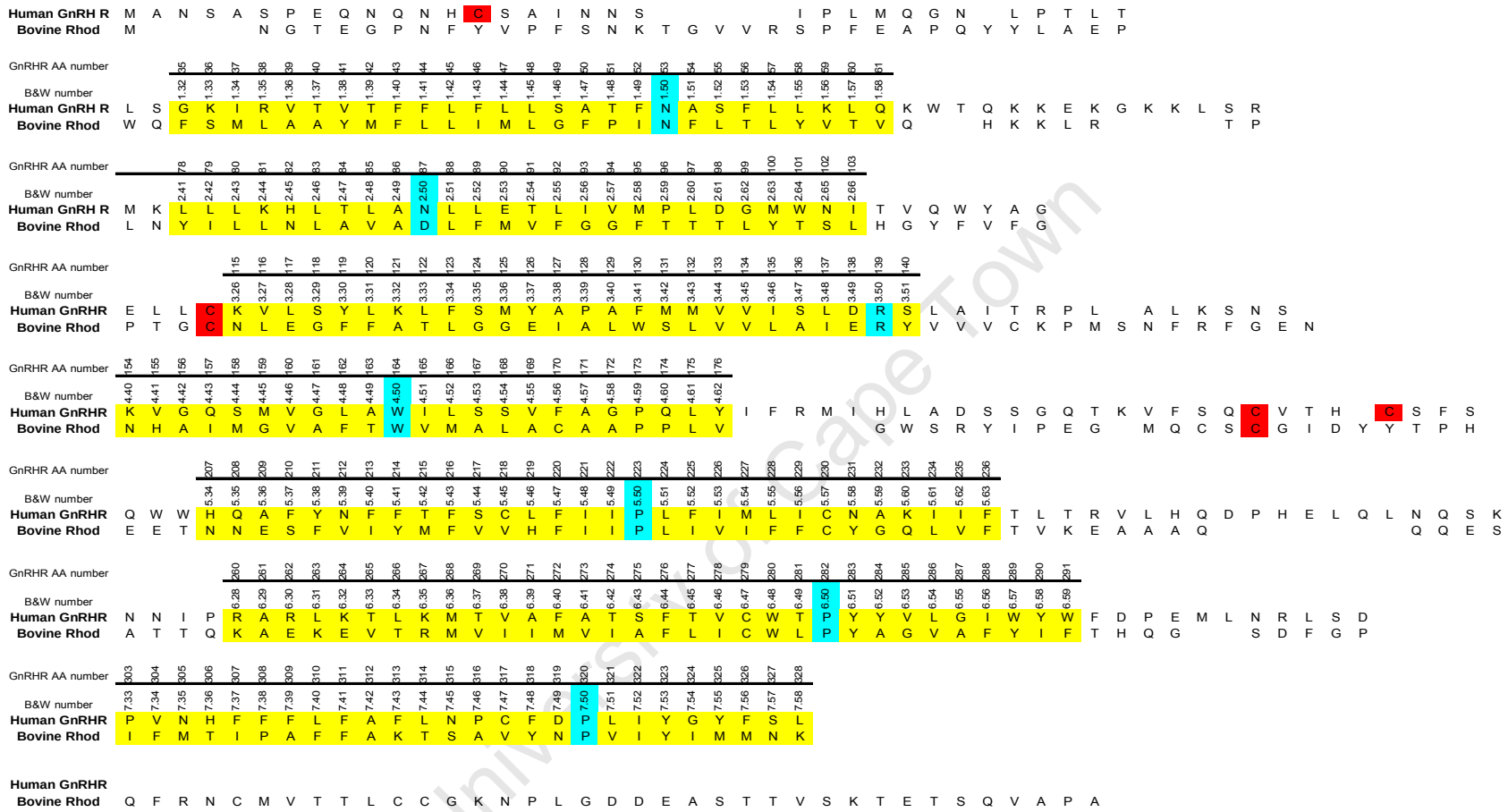


Figure 1.4. Sequence alignment of the human GnRH receptor and bovine rhodopsin. Residues highlighted in yellow represent approximate TM regions. Blue highlighted residues are the most highly conserved residues in each TM domain. Cysteines in red represent residues that form disulfide bridges (see text). The GnRH receptor amino acid (AA) numbers and the corresponding Ballesteros and Weinstein (B&W) numbers are indicated in the TM domains.

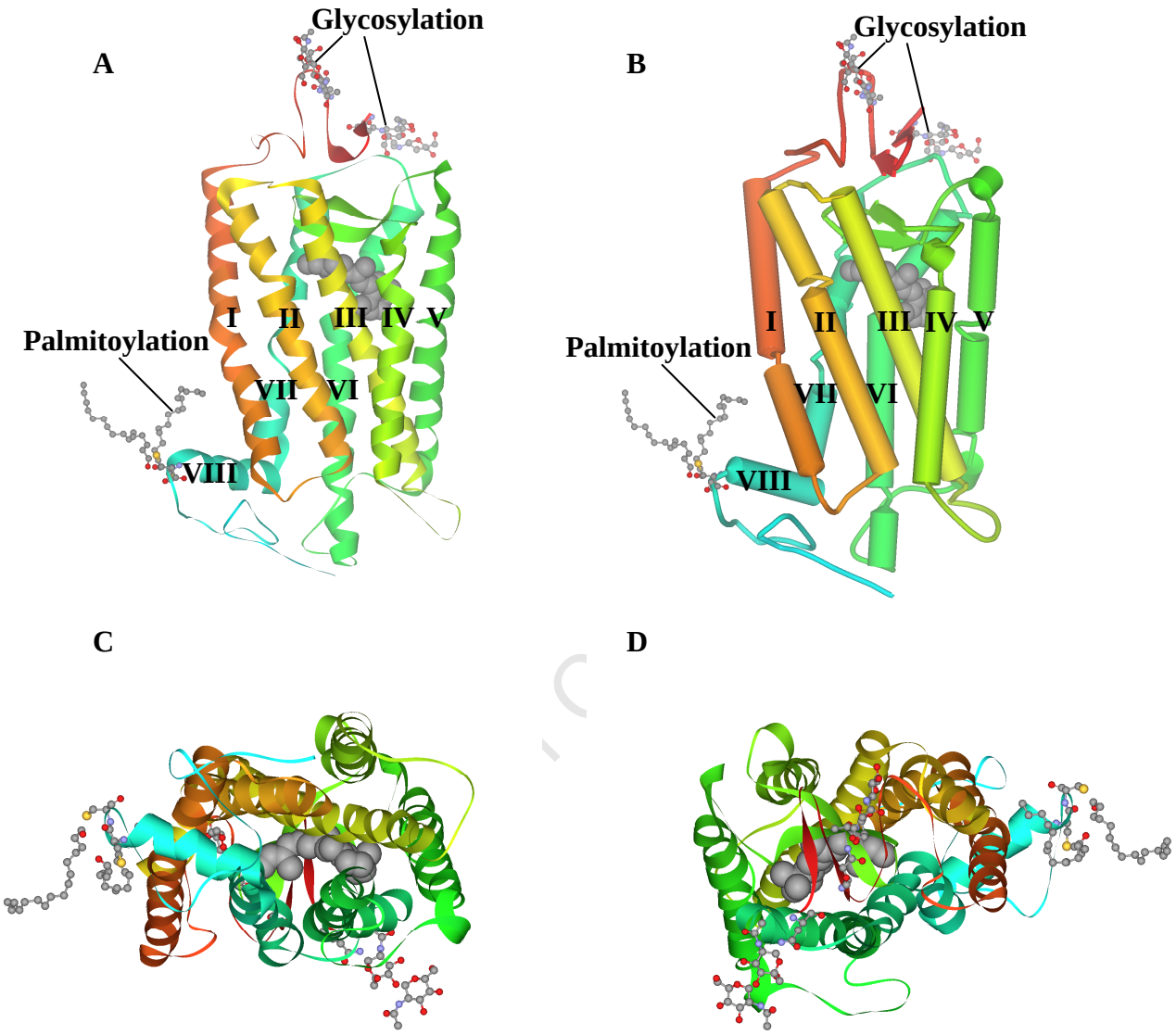


Figure 1.5. Crystal structure of bovine rhodopsin. The crystal structure of rhodopsin is represented in ribbon (A) and schematic (B) forms, as viewed from within the plane of the membrane. TM numbering (in Roman numerals), glycosylation and palmitoylation modifications are indicated. Rhodopsin's ligand, 11-*cis*-retinal, is represented in grey. The schematic figure allows easier identification of the four β-sheets formed by the N-terminal and ECL2 regions, which form a layered roof over the centre of the TM domains. Rhodopsin's structure as viewed from the intracellular (C) and extracellular (D) surfaces are also shown. Figures drawn from the 1U19 pdb file (Okada et al., 2004) using DS Visualiser software (Accelrys).

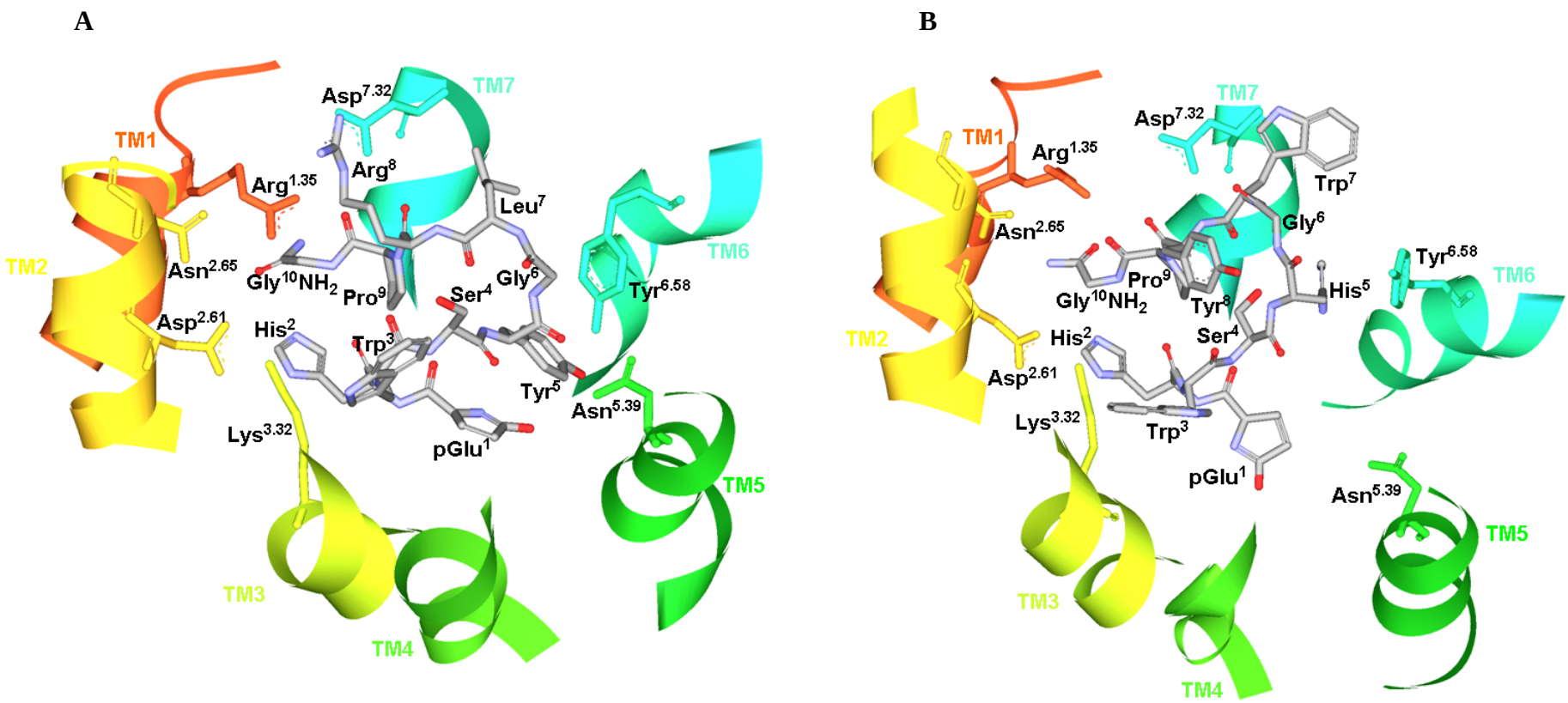


Figure 1.6. Ligand docking models of the human GnRH receptor. A, GnRH I docked at the GnRH receptor. GnRH I is proposed to make the following interactions: $PGlu^{1}$ interacts with $Asn^{5.39}$; His^{2} interacts with $Asp^{2.61}$ and $Lys^{3.32}$; Tyr^{5} interacts with $Tyr^{6.58}$; Arg^{8} interacts with $Asp^{7.32}$; Pro^{9} and the C-terminal glycinamide interact with $Arg^{1.35}$ and $Asn^{2.65}$. B, GnRH II docked at the GnRH receptor. GnRH II is proposed to make the following interactions: His^{2} interacts with $Asp^{2.61}$ and $Lys^{3.32}$; Pro^{9} and the C-terminal glycinamide interact with $Arg^{1.35}$. $Asp^{7.32}$ confers specificity for GnRH I and does not contribute to GnRH II binding. The molecular models of the GnRH receptor are based on homology modelling of rhodopsin (1U19). The figures were prepared using DS Visualiser (Accelrys) and represent the receptor as viewed from the extracellular surface.

1.4 GnRH receptor activation

1.4.1 Theoretical models of GPCR activation: the Ternary Complex Model

Important early experiments that shaped our understanding of GPCR activation were incorporated into theoretical models which are useful to explain and predict receptor behaviour. One of the earliest models was termed the ternary complex model (TCM) (De Lean et al., 1980). This model states that receptors exist in an equilibrium of two agonist affinity states, a high affinity and low affinity state. The model proposes that the high affinity state of the receptor is the result of a ternary complex association of the agonist, receptor and G protein whereas the low affinity state is characterised by the absence of G proteins in the complex. Alterations in any of the three components of the ternary complex shift the equilibrium of the two receptor agonist affinity states, according to the laws of mass action. This model is useful in understanding the importance of receptor-G protein interactions in receptor pharmacology and provides an explanation for the observed differences in the pharmacological profile and constitutive activity of a receptor in different tissues where the relative receptor: G protein ratios may vary.

The discovery that mutation of specific residues in a receptor could induce constitutive activity and resulted in a high affinity agonist binding state of the receptor, without the presence of G proteins, was not consistent with the TCM. Thus the TCM was refined and denoted the extended ternary complex model (ETCM) (Samama et al., 1993). In this model, the two states, characterised by differences in agonist binding affinity, were termed R and R*, where R represents the low affinity inactive receptor conformation and R* represents the high affinity active (G protein-activating) receptor conformation. An isomerisation constant, J, was introduced which provides a measure of the proportion of receptors in R and R* and is influenced by the inherent ability of the receptor to change conformation. Receptor mutations that induce constitutive activity are proposed to ease the transition of receptors from R to R* thereby shifting the equilibrium of receptors to a higher proportion in the active conformation. In this model, agonists bind with higher affinity to R* shifting the equilibrium of receptors towards the active conformation and promoting a functional response. In contrast, inverse agonists bind

with higher affinity to R altering the receptor conformational equilibrium in favour of the inactive conformation and decreasing basal receptor activity. Neutral antagonists bind with equal affinity to both R and R* and thus do not change the receptor equilibrium and do not produce a ligand-induced functional signalling response.

Emerging experimental evidence has revealed some limitations of the ETCM (Kenakin, 2002), which will be discussed in more detail in section 1.5.6. However, this model highlights an important aspect of receptor activation. Specifically, that receptor activation requires a conformational transition from an inactive to an active conformation. This transition is associated with a specific energetic barrier that can be lowered by mutation-induced disruption of receptor intramolecular constraining interactions (Kobilka, 2007). The identification of a role for a number of highly conserved motifs in receptor activation has led to the proposal of a common mechanism of activation utilised by all rhodopsin family GPCRs (Karnik et al., 2003; Schwartz et al., 2006). Thus much of the current understanding of GnRH receptor activation is inferred from experiments with other rhodopsin family GPCRs, such as rhodopsin and the β_2-AR. In this section, key early experiments and recent innovative work providing insight into GPCR activation are outlined. Wherever possible, experimental evidence specific to the GnRH receptor is provided.

1.4.2 Conformational changes associated with receptor activation

Experimental evidence and analysis of conserved structural motifs suggests that GPCRs undergo similar structural rearrangements upon activation (Karnik et al., 2003; Schwartz et al., 2006). These conformational changes associated with receptor activation are proposed to involve an outward movement of TM6 relative to TM3 and an outward movement of TM7, at the cytoplasmic end of the receptor (see Fig.1.7) (Gether, 2000; Karnik et al., 2003; Lu et al., 2002; Okada et al., 2001; Schwartz et al., 2006). This facilitates exposure of receptor epitopes responsible for G protein binding and activation.

These conformational changes are suggested by various biophysical and biochemical experiments in several GPCRs (Meng and Bourne, 2001). Site-directed spin-labelling (SDSL) studies with rhodopsin suggest that light activation induces an outward rotational movement of TM6 relative to TM3 at the intracellular end of the receptor (Farrens et al., 1996). A similar conformational rearrangement upon receptor activation was detected in the β_2-AR using environmentally sensitive fluorophores (Ghanouni et al., 2001b; Kobilka, 2002). The absolute requirement of this conformational change for receptor activation is underlined by the inability of receptors with disulfide bridges or engineered metal-ion binding sites between the intracellular ends of TM3 and TM6 to induce G protein activation (Cai et al., 1999; Meng and Bourne, 2001; Sheikh et al., 1999).

Furthermore, SDSL and activation-dependent generation of intramolecular disulfide bridges suggest an outward movement of TM7 upon receptor activation (Yang et al., 1996; Yu et al., 1999). Additional evidence for TM7 exposure at the intracellular end of the receptor upon receptor activation is provided by the activation state-dependent binding of an antibody to a TM7 epitope including a portion of the NPxxY motif (Abdulaev and Ridge, 1998).

Thus receptor activation involves an outward rotational movement of TM6 and to a lesser degree the outward movement of TM7, relative to TM3, at the intracellular surface of the receptor (Lu et al., 2002; Palczewski, 2006; Schwartz et al., 2006). The outward motion of TM6 and TM7 at the intracellular end of the TM bundle is coupled with an inward movement of these helices towards TM3 at the extracellular surface of the membrane. This was demonstrated by the creation of a metal ion binding site between residues at the extracellular end of TM3, TM6 and TM7 that induced receptor activation (Elling et al., 2006). Consultation of the crystal structure of the inactive state of rhodopsin revealed that a metal ion binding site could not be accommodated by these residues without an inward movement of TM6 and TM7. The authors propose a model of receptor activation where proline residues within the helices act as hinges for the resulting “see-saw” movement of TM6 and TM7 upon receptor activation (Elling et al.,

2006; Schwartz et al., 2006). However, the magnitude of the helical rearrangements predicted by biochemical and biophysical experiments may have been overestimated as a recent photoactivated crystal structure of rhodopsin revealed only minor conformational changes (Salom et al., 2006).

1.4.3 Highly conserved GPCR residues or motifs facilitate receptor activation

A number of highly conserved GPCR residues and motifs assist in mediating the receptor conformational changes outlined above. These residues include several highly conserved proline residues and the D/NPxxY, E/DRY and CWxP motifs (Gether, 2000; Schwartz et al., 2006) (see Fig.1.8).

1.4.3.1 The conserved prolines Pro$^{6.50}$ and Pro$^{7.50}$

Proline residues have a significant influence on receptor structure as they induce disruptions within the 7TM helices and are often associated with a bend or kink at that region within the helix (Ballesteros et al., 2001b). Thus these residues play an important role in directing the overall receptor structure (see section 1.3). Two proline residues in TM6 and TM7, Pro$^{6.50}$ and Pro$^{7.50}$, are highly conserved amongst GPCRs (Mirzadegan et al., 2003). Furthermore, the essential role of these proline residues is illustrated by the disruption of receptor folding and function when they are mutated in a number of receptors (Sansom and Weinstein, 2000). In addition to the important effects on receptor folding, proline residues are proposed to act as flexible hinges that facilitate the conformational changes associated with receptor activation, particularly the movement of TM6 and TM7 (Elling et al., 2006; Sansom and Weinstein, 2000; Schwartz et al., 2006). The presence of the highly conserved prolines within the GnRH receptor sequence suggests that the GnRH receptor structure is compatible with this proposed mechanism of activation (see Fig.1.4).

1.4.3.2 The CWxP motif and the rotamer toggle switch

Movement of the proline hinges is modulated by intramolecular and interhelical interactions of the residues above and below the relevant prolines in the helix. Cys$^{6.47}$, Trp$^{6.48}$ and Pro$^{6.50}$ constitute the highly conserved CWxP motif in TM6 of rhodopsin

family GPCRs (Mirzadegan et al., 2003; Schwartz et al., 2006). In the β_2-AR, mutation of $Cys^{6.47}$ to Ser, which has a similar distribution of side chain rotamer configurations in the helix, demonstrates receptor behaviour comparable with the wildtype receptor (Shi et al., 2002). However, mutation of $Cys^{6.47}$ to Thr, for which the population of side chain configurations in the helix differs, results in a receptor that exhibits constitutive activity. This suggests that the rotamer configuration of the $Cys^{6.47}$ side chain plays an important role in receptor activation (Shi et al., 2002). Furthermore, the biased monte carlo technique of conformational memories indicates that the rotamer conformation of $Cys^{6.47}$ is coupled with that of $Trp^{6.48}$ and $Phe^{6.52}$. The co-ordinated position of these highly conserved residues is proposed to modulate the angle of the TM6 proline kink and thus the movement of TM6, suggesting that these residues form part of a rotamer toggle switch which facilitates receptor activation (Schwartz et al., 2006; Shi et al., 2002).

Nuclear Magnetic Resonance (NMR) experiments suggest that $Trp^{6.48}$ also undergoes a shift in orientation upon light activation of rhodopsin which is consistent with the rotamer toggle switch proposed in the β_2-AR (Crocker et al., 2006). Furthermore, $Trp^{6.48}$ is implicated in the regulation of receptor activation in several other GPCRs (Joubert et al., 2002; Lu et al., 2004; Marie et al., 2001). Thus this proposed mechanism activation switch may be more broadly applicable to the rhodopsin family GPCRs (Singh et al., 2002).

In the GnRH receptor, mutation of $Trp^{6.48}$ to Ser or Ala and $Cys^{6.47}$ to Tyr or Ala resulted in significantly decreased receptor expression levels (Chauvin et al., 2000; Chauvin et al., 2001; Janovick et al., 2002; Lu et al., 2007). This result suggests that these residues participate in important intramolecular interactions that stabilise receptor structure and facilitate the correct folding of the GnRH receptor. Following rescue of $Trp^{6.48}$- and $Cys^{6.47}$-mutated receptor expression with the small molecule antagonist IN3, IP signalling could be detected (Janovick et al., 2002; Lu et al., 2007). Constitutive activity was not observed, but the absence of complete data for the calculation of signalling efficiencies of these mutant receptors prevents definitive conclusions regarding the role

of these residues in receptor activation (Janovick et al., 2002; Lu et al., 2007). Nevertheless, an interesting observation is that the Cys$^{6.47}$Ala and Cys$^{6.47}$Tyr mutant receptors had increased affinity for GnRH II, but not GnRH I. As Cys$^{6.47}$ is not a direct GnRH II binding site, this suggests that Cys$^{6.47}$ mutation alters the ligand binding pocket by receptor conformational changes (Lu et al., 2007). This suggests that Cys$^{6.47}$ is also spatially positioned in the GnRH receptor to contribute to receptor conformational or structural rearrangements, which is consistent with the toggle switch model.

1.4.3.3 The D/NPxxY motif

A second set of highly conserved residues cluster around Pro$^{7.50}$ and form the D/NPxxY motif. The Asp/Asn$^{7.49}$ and Tyr$^{7.53}$ on either side of the proline are proposed to participate in distinct sets of intramolecular interactions that modulate the angle of the proline kink in TM7 and thus TM7 movement upon receptor activation (He et al., 2001; Sansom and Weinstein, 2000).

Asp/Asn$^{7.49}$ of the D/NPxxY motif is proposed to undergo a conformational rearrangement and participate in discrete intramolecular interactions in the inactive and active states of the receptor. In the inactive conformation of rhodopsin, Asn$^{7.49}$ forms part of a hydrogen bonding network with Asn$^{1.50}$ and Asp$^{2.50}$ which connects TM1, TM2 and TM7 (Li et al., 2004; Palczewski et al., 2000). Asp$^{2.50}$ is central to this interactive network, interacting with Asn$^{1.50}$ by direct hydrogen bonding and with Asn$^{7.49}$ via a bridging water molecule. Asn$^{7.49}$ also participates in interactions with residues in H6 in the inactive state which may be relevant to receptor activation (Li et al., 2004; Okada et al., 2004; Urizar et al., 2005). In the thyrotropin receptor (TSHR), mutation-induced disruption of an interaction between Asn$^{7.49}$ and Thr$^{6.43}$ and Asp$^{6.44}$ in H6 induces constitutive activity (Urizar et al., 2005). The crystal structure of rhodopsin reveals an analogous interaction is present in rhodopsin and thus this may represent a more general mechanism for GPCR activation (Urizar et al., 2005).

Due to the low resolution of the active MetaII crystal structure of rhodopsin, the interaction partners for Asn$^{7.49}$ in the active state of GPCRs are less clear. (Salom et al.,

2006). However, site-directed mutagenesis experiments and molecular modelling in the TSHR and the Histamine H_1 receptor suggest that $Asn^{7.49}$ may interact directly with $Asp^{2.50}$ and a second ionic counterpart that could be $Arg^{3.50}$ of the DRY motif in the active state of GPCRs (Bakker et al., 2008; Urizar et al., 2005).

The GnRH receptor has undergone a reciprocal mutation at positions 2.50 and 7.49 (Flanagan et al., 1999; Zhou et al., 1994). As discussed in section 1.3.1, analysis of the effects of mutations at these positions on GnRH receptor expression and ligand binding affinity suggest that a similar set of interactions between $Asn^{1.50}$ and $Asn^{2.50}$ and $Asp^{7.49}$ occur in the inactive conformation of the GnRH receptor (Flanagan et al., 1999; Zhou et al., 1994). Mutation of $Asp^{7.49}$ to Ala in the GnRH receptor resulted in a coupling efficiency of 0.7% of the wildtype receptor value, as measured by IP assays (Flanagan et al., 1999). This reveals that $Asp^{7.49}$ also plays a critical role in receptor activation of the GnRH receptor. Interestingly, when the configuration of the 2.50 and 7.49 residues observed in the GnRH receptor was introduced into the TSHR, the TSHR exhibited decreased constitutive activity (Urizar et al., 2005). Thus perhaps this feature facilitates a more constrained inactive receptor conformation in the GnRH receptor.

The side chain of $Tyr^{7.53}$, on the other side of the proline kink, participates in a different set of intramolecular interactions. In the inactive state of rhodopsin, $Tyr^{7.53}$ forms an intramolecular constraining interaction with $Phe^{7.60}$ in H8. Mutation-induced disruption of this interaction in rhodopsin results in MetaII formation (Fritze et al., 2003). The suggestion that these residues participate in a constraining intramolecular interaction in the inactive state of the receptor is strengthened by the observation that a disulfide bond created between $Tyr^{7.53}$ and $Phe^{7.60}$ prevented MetaII formation (Fritze et al., 2003).

$Tyr^{7.53}$ may also play a role in stabilising the active conformation of GPCRs. Mutation of $Tyr^{7.53}$ in the $5HT_{2C}$ serotonin receptor displayed diverse phenotypes, ranging from uncoupled to high constitutive activity, depending on the nature of the side chain substitution at that position (Prioleau et al., 2002). This suggests that $Tyr^{7.53}$ may be involved in the receptor conformational switch from inactive to active conformations.

Despite the absence of H8, mutation of $Tyr^{7.53}$ to Ala in the GnRH receptor abolished G protein activation, as measured by IP assays (Arora et al., 1996). This suggests that $Tyr^{7.53}$ is also important for GnRH receptor activation. Additionally, $Tyr^{7.53}$ to Ala mutation in the GnRH receptor differentially affects the binding of GnRH I and GnRH II (Lu et al., 2005). As the cytoplasmic position of $Tyr^{7.53}$ prevents its direct interaction with GnRH II, this effect is more likely due to the ability of this residue to modulate receptor conformation, as observed with the $Cys^{6.47}$ mutation.

1.4.3.4 The E/DRY motif and the ionic lock

The highly conserved E/DRY motif is a key element of GPCR activation (Flanagan, 2005; Gether, 2000; Okada et al., 2001; Rovati et al., 2007). In the crystal structure of inactive rhodopsin, $Arg^{3.50}$ interacts with the neighbouring $Glu^{3.49}$ and $Glu^{6.30}$ positioned in ICL3 below TM6 (Li et al., 2004; Okada et al., 2004; Palczewski, 2006). A charge neutralising mutation of $Glu^{3.49}$ to Gln induces increased constitutive activity at opsin, the form of rhodopsin which lacks the presence of the inverse agonist 11-*cis*-retinal (Acharya and Karnik, 1996). Additionally, SDSL experiments reveal that this mutation results in helical rearrangements that are associated with rhodopsin activation (Kim et al., 1997). Similarly, in the β_2-AR, receptor mutants with charge-neutralising mutations of $Asp^{3.49}$ or $Glu^{6.30}$ exhibited constitutive receptor activity (Ballesteros et al., 2001a). Using methanethiosulfonate (MTSEA) assays which assessed the accessibility of $Cys^{6.47}$, it was possible to infer that these $Asp^{3.49}$ or $Glu^{6.30}$ mutations also induced conformational rearrangements in the helices of the β_2-AR (Ballesteros et al., 2001a). This led to the proposal that the network of interactions involving the triad, $Arg^{3.50}$, $Asp^{3.49}$ and $Glu^{6.30}$, constitute an "ionic lock" or constraining interaction that prevents helical movement and GPCR activation (Ballesteros et al., 2001a).

While $Asp^{3.49}$ and $Arg^{3.50}$ are highly conserved amongst rhodopsin family GPCRs, $Glu^{6.30}$ is not (Mirzadegan et al., 2003). Nevertheless, mutation of $Asp^{3.49}$ and $Arg^{3.50}$ results in alterations in the signalling capacity and conformation of a wide variety of

GPCRs, suggesting that they have a conserved function in the regulation of receptor activation (Flanagan, 2005; Rovati et al., 2007). In the GnRH receptor, mutation of $Asp^{3.49}$ to Asn resulted in an enhancement of receptor signalling efficiency, suggesting that this mutation disrupts a constraining interaction (Ballesteros et al., 1998). Computational molecular modelling of the GnRH receptor supports the feasibility that this constraining interaction consists of $Asp^{3.49}$ and $Arg^{3.50}$ (Ballesteros et al., 1998).

In addition to interactions that constrain the inactive conformation of the receptor, $Arg^{3.50}$ is also implicated in stabilising the active state of GPCRs. In the active state, $Arg^{3.50}$ is proposed to interact with $Asp^{2.50}$ and $Asn^{7.49}$ (Bakker et al., 2008; Urizar et al., 2005). This interaction is also predicted to occur in the active state of the GnRH receptor (Ballesteros et al., 1998). This suggestion is consistent with the observation that mutations of both $Arg^{3.50}$ and $Asp^{7.49}$ in the GnRH receptor significantly compromise receptor activation, as measured by IP signalling (Arora et al., 1997; Flanagan et al., 1999).

$Tyr^{3.51}$ in the E/DRY motif is the least conserved residue of this motif in rhodopsin family GPCRs (Mirzadegan et al., 2003; Rovati et al., 2007). Indeed, $Tyr^{3.51}$ mutation in a range of GPCRs is associated with either the absence or only minor affects on receptor signalling suggesting that this conserved residue is not required for receptor activation (Rovati et al., 2007). The GnRH receptor has a serine at position 3.51 and thus exhibits a DRS, as opposed to the typical DRY, motif. Mutation of $Ser^{3.51}$ to Ala in the GnRH receptor did not affect IP signalling, consistent with the suggestion that this residue does not contribute to receptor activation (Arora et al., 1997).

1.4.4 Summary

An underlying implication of the conserved structure of GPCRs is a common mechanism of activation (Karnik et al., 2003; Schwartz et al., 2006). GPCR activation is proposed to involve the disruption of a set of constraining intramolecular interactions or molecular switches, which facilitates receptor helical rearrangements, particularly of TM3, TM6 and TM7 (Meng and Bourne, 2001). These conformational changes assist

the formation of a new set of intramolecular interactions that stabilise the active state of the receptor. The conformational transition involved in receptor activation is guided by a set of key highly conserved residues, including the CWxP, D/NPxxY and E/DRY motifs and facilitated by the presence of highly conserved proline residues, $Pro^{6.50}$ and $Pro^{7.50}$, which act as hinges for the conformational changes (Karnik et al., 2003; Schwartz et al., 2006). Analysis of the effects of mutation of these key residues in the GnRH receptor on receptor activation are consistent with the suggestion that this proposed mechanism of activation is utilised by the GnRH receptor. Nevertheless, currently, no mutation of the GnRH receptor has been sufficient to induce constitutive activity (Lu et al., 2007; Millar et al., 2004; Myburgh et al., 1998b). This may reflect an increased set of constraining interactions in the GnRH receptor and/or that the GnRH receptor requires the directive action of new intramolecular interactions in order to attain the active state rather than "springing" into the active state as observed with other receptors (Lu et al., 2007).

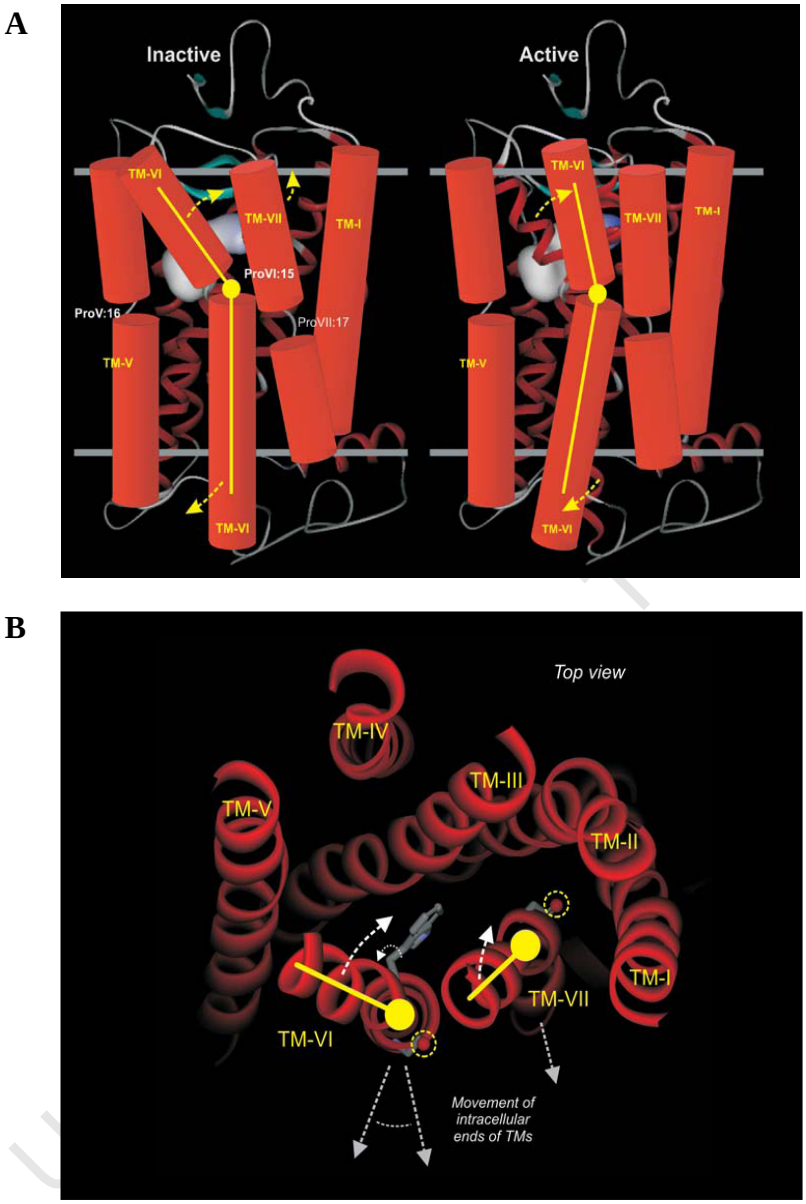


Figure 1.7. GPCR conformational changes induced upon receptor activation. Proposed conformational changes that GPCRs undergo upon receptor activation as viewed from within the plane of the membrane (A) and from the extracellular surface (B). GPCRs are proposed to undergo a conformational change upon receptor activation involving an outward rotational movement of TM6, and to a lesser degree TM7, relative to TM3 at the intracellular end of the receptor. This is coupled with an inward movement of these helices at the extracellular end of the TM domains. This "see-saw" movement is facilitated by proline residues (highlighted with yellow dashed circles), which act as hinges for this mechanical event about the indicated pivot points (represented with yellow balls). The rotamer conformation of $Trp^{6.48}$ (indicated in grey in B) of the CWxP motif facilitates these movements by the rotamer toggle switch (see text). Figure taken from Schwartz et al. (Schwartz et al., 2006).

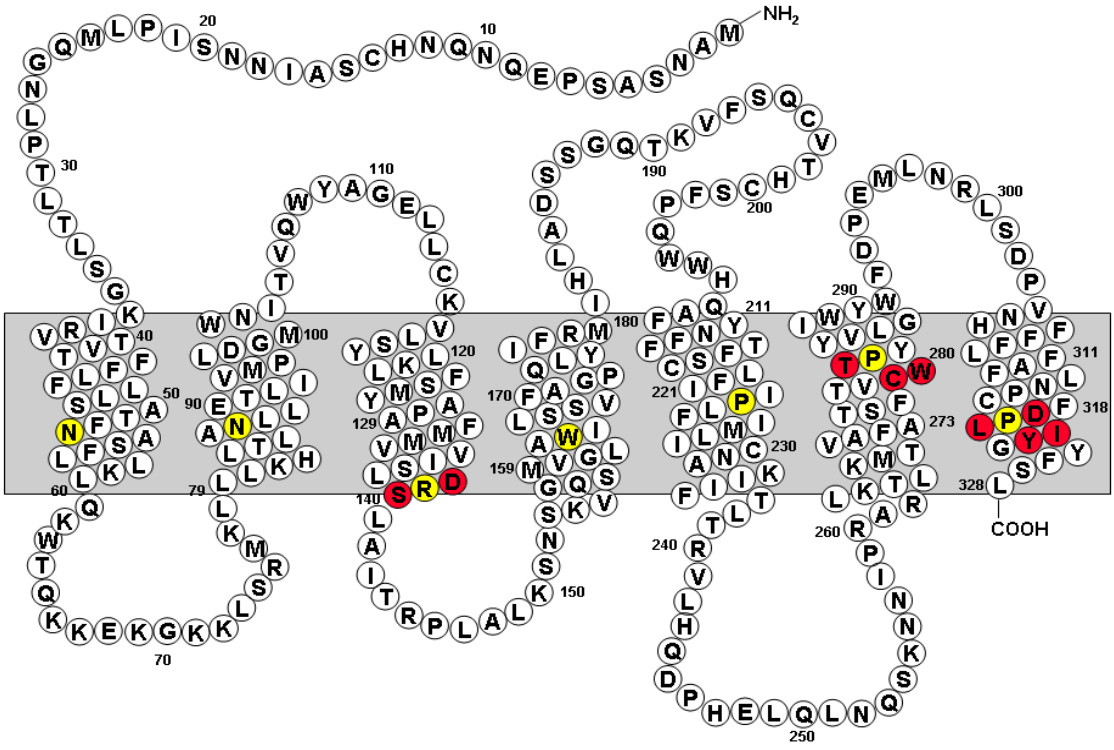


Figure 1.8. Highly conserved GPCR motifs facilitating receptor activation mapped onto the GnRH receptor sequence. This figure is a two-dimensional representation of the human GnRH receptor showing the TM domains connected by the ECLs and ICLs. The most highly conserved residue in each TM domain is indicated in yellow. Highly conserved residues important for receptor activation, specifically the DRS, CWxP and DPxxY motifs, are in red.

1.5 Ligand-induced receptor activation and signalling

1.5.1 Agonist binding induces receptor activation

As described above, receptor activation involves the disruption of key intramolecular interactions that facilitate receptor conformational changes. Thus the question arises: how does agonist binding induce receptor activation? Due to the unusual covalent linkage of rhodopsin's ligand within the receptor, this model system is less suitable to understand the complex process of agonist binding at the GnRH receptor. Thus experimental data describing the binding of diffusible agonists at the β_2-AR will be discussed in this section. This information and experimental work specific to the GnRH receptor are incorporated into a hypothesis regarding the probable process of agonist binding at the GnRH receptor.

Current thinking is that agonists perform two functions that facilitate receptor activation (Kobilka, 2007; Kobilka and Deupi, 2007). Firstly, agonists facilitate the disruption of intramolecular interactions or molecular switches that constrain the receptor in the inactive state. Experimental evidence for this role of agonists is provided by a method that was able to measure disruption of the intramolecular interactions constituting the ionic lock and the rotamer toggle switch in the β_2-AR (Yao et al., 2006). Specifically, it was shown that disruption of these two intramolecular switches correlated with ligand efficacy, where full agonists disrupted both switches while antagonists did not disturb either set of interactions (Yao et al., 2006).

The second role of ligands is that they create a bridge between opposing helices that stabilises the active conformation of the receptor (Kobilka and Deupi, 2007). As discussed, the proposed receptor conformational changes involve a see-saw motion of the 7TM helices where the extracellular ends move inward (coupled with an outward motion of the intracellular ends) (Schwartz et al., 2006). Experimental support for this role was provided following creation of a metal ion binding site in the β_2-AR by mutation of specific residues in TM3, TM6 and TM7. This mutant receptor could be activated by the bridging action of metal ions which are proposed to stabilise the inward

active position of the extracellular ends of the TM helices (Elling et al., 2006). Thus agonist-induced receptor activation involves the disruption of intramolecular interactions which promotes formation of the active conformation of the receptor that is stabilised by the bridging action of the agonist (Kobilka and Deupi, 2007).

The energetic feasibility of the conformational transition from inactive to active receptor is facilitated by the formation of a new set of favourable interactions between the agonist and receptor. It is suggested that these agonist-receptor interactions form by a sequential multi-step process. Initial agonist-receptor contacts are proposed to facilitate receptor conformational changes that enable subsequent agonist-receptor interactions to occur. This is supported by evidence that agonist-receptor contacts at one end of the β_2-AR synergistically contribute to the binding of agonist-receptor contacts at a remote region of the receptor (Del Carmine et al., 2004). β_2-AR agonists comprise two domains, a catechol ring and a tail, which are proposed to bind to opposing helices within the β_2-AR. Three serine residues ($Ser^{5.42}$, $Ser^{5.43}$ and $Ser^{5.46}$) in TM5 contribute to the binding of functional groups on the catechol ring. The loss in binding affinity at receptors with mutations of these serine residues was smaller when there were less functional groups in the catechol tail (Del Carmine et al., 2004). Considering the limited flexibility of the small molecule catecholamine agonist, the authors concluded that this synergism is the result of receptor conformational changes initiated by preceding agonist-receptor interactions which thereby facilitate the formation of subsequent agonist-receptor contacts (Del Carmine et al., 2004). Thus agonist binding is suggested to occur by an induced-fit mechanism. Furthermore, the sequential nature of the agonist binding process suggests that multiple intermediate receptor conformations should be formed. Indeed, using a β_2-AR which is fluorescently labelled to assess receptor conformational changes in real time, the presence of consecutive distinct receptor active conformations in response to agonists is observed (Swaminath et al., 2004).

Agonist binding at the GnRH receptor is expected to follow a similar multi-step pattern. However, in contrast to the β_2-AR, where the small molecule agonists have limited

conformations, the larger, more flexible GnRH peptides add additional complexity to the agonist binding process at the GnRH receptor. While information on the mechanism of GnRH I binding is limited, a few key observations allow postulation of the mechanism of binding. Firstly, analysis of GnRH I-receptor contacts (see section 1.3) reveals that the GnRH I C-terminal residues, Arg^{8}, Pro^{9} and the $Gly^{10}NH_2$, interact predominantly with TM1, TM2 and extracellular surface of the receptor at $Asp^{7.32}$ in ECL3 (see Fig.1.9). Substitutions at these positions in the ligand decrease ligand binding affinity revealing an important role for the C-terminal residues in receptor binding (Millar et al., 2004; Sealfon et al., 1997). Furthermore, the interaction of Arg^{8} with $Asp^{7.32}$ is proposed to represent a transient interaction that facilitates configuration of the βII'-type turn of GnRH I (Fromme et al., 2001). In contrast, the N-terminal residues of GnRH I, $pGlu^{1}$, His^{2} and Trp^{3} are proposed to interact mainly with receptor residues within TM3 and TM5 (see Fig.1.9). Mutations of the proposed receptor contact residues for this N-terminal region of the ligand have illustrated an important role for these residues in GnRH receptor activation (Millar et al., 2004; Sealfon et al., 1997). Furthermore, substitutions of these N-terminal residues with D-amino acids result in the generation of GnRH receptor antagonists (Sealfon et al., 1997). This is consistent with the observed role of agonist contacts with TM3, TM5 and TM6 in β_2-AR activation (Swaminath et al., 2004). Consolidating these experimental observations, I propose the following simplistic scheme for the binding of GnRH I to the GnRH receptor. The C-terminal region of GnRH I makes initial contacts with the receptor, serving to anchor the ligand to the receptor and promoting formation of the high affinity conformation of the ligand. This facilitates the insertion of the N-terminal region of GnRH I into the 7TM helices for interaction with TM3 and TM5. The binding of GnRH II is likely to follow a similar pattern considering the conserved nature of the N- and C-termini. However, its differential interaction with $Asp^{7.32}$ is likely to facilitate slightly different receptor interactions.

1.5.2 Intermediate receptor conformations induced upon agonist binding have functional relevance

The sequential mechanism of agonist binding predicts an important aspect of receptor structure and behaviour. Specifically, that GPCRs do not simply switch between two conformations, the inactive and active state, but rather that receptors can assume multiple conformations. Interestingly, some of the multiple intermediate receptor conformations initiated by agonist binding may have distinct functional significance and this results in initiation of different downstream signalling pathways which are activated in a sequential manner.

An experimental illustration is provided by analysis of agonist-induced β_2-AR conformational changes in real time using fluorescence microscopy (Swaminath et al., 2004). In response to the agonist, norepinephrine (NE), two conformational components were observed at the receptor, a set of rapid conformational changes followed by a set of slower conformational changes. In contrast, the agonist dopamine (DA), which differs from NE by a single hydroxyl group, induced only rapid conformational changes at the receptor. Analysis of the differences in signalling properties of the two agonists revealed that, while DA is strongly coupled to G protein signalling, its capacity to mediate receptor internalisation is significantly compromised compared with NE (Swaminath et al., 2004). The authors propose that NE functional groups bind to the receptor in a sequential manner inducing a series of receptor conformational intermediates. The first set of rapid conformational changes induces a conformation that facilitates G protein activation. This is followed by a second set of conformational changes initiated by the hydroxyl group which distinguishes it from DA. This set of conformational changes has a high energetic barrier and thus occurs at a slower rate and results in a conformation that facilitates receptor internalisation (Swaminath et al., 2004). Thus NE initiates a series of conformational changes that facilitate sequential activation of the G protein and internalisation pathways.

1.5.3 Ligand-induced selective signalling (LiSS)

The work described above highlights an important aspect of agonist-induced receptor activation, notably, that agonist structure has the capacity to direct the conformation of the receptor and thus select the downstream signalling pathway(s) activated. In the above example, DA was only able to activate G protein signalling. However, NE, which has an additional hydroxyl group, was able to initiate activation of both G protein and internalisation pathways. This suggests that compared with DA, NE stabilises a different subset of receptor conformations that can be recognised by both G proteins and the internalisation machinery of the cell.

The concept of LiSS refers to the ability of structurally distinct ligands to induce different conformations at the same receptor that thereby determine the capacity of the receptor to signal to different downstream signalling pathways (see Fig.1.10). There are a number of terms used to denote this concept including agonist-directed trafficking and functional selectivity (Kenakin, 1995; Kenakin, 2001; Kenakin, 2003; Urban et al., 2007). In this review, it will be referred to as LiSS as this term considers the role of the ligand in the selection of the signalling pathway, but is not biased by historical classifications of ligands as agonists or antagonists (Millar et al., 2004; Millar et al., 2008). This is important, because, as discussed below, certain ligands previously classified as antagonists of a particular G protein signalling pathway, have demonstrated the capacity to activate other signalling pathways.

There are several predictions from LiSS that can be investigated experimentally and thus provide evidence for its existence (Perez and Karnik, 2005; Urban et al., 2007). The first is that structurally distinct ligands facilitate induction of different receptor conformations. This feature of LiSS is supported by investigations using experimental techniques that directly probe the receptor conformation (Ghanouni et al., 2001a). Indeed, two agonists of the β_2-AR, isoproterenol and DA, induced different fluorescence lifetime distributions of the fluorescently-labelled β_2-AR, reflecting their capacity for induction of different receptor conformational states (Ghanouni et al., 2001a). The

ability of different ligands to induce different receptor conformations is also inferred from studies describing the differential capacity of structurally distinct ligands to disturb the rotamer toggle switch and ionic lock (Swaminath et al., 2005; Yao et al., 2006).

A second prediction of LiSS is that different receptor conformations have differential capacities to activate downstream signalling pathways. Thus receptor mutations that disrupt intramolecular interactions, and thus alter the receptor's conformation, may alter the signalling capacity of the receptor at one signalling pathway, but not another. Furthermore, different ligands, which stabilise distinct receptor conformations, should exhibit reversals of potencies at different downstream signalling pathways within the same experimental or cellular context.

Several examples examining G protein signalling pathways of GPCRs support these described features of LiSS. Examples involve identification of the reversal or change in the relative efficacy of ligands at different G protein pathways. In the 5-HT_{2C} serotonin receptor, which couples to both the G_q and G_i protein classes, a group of agonists with comparable abilities to activate G_q, displayed differences in their abilities to activate G_i signalling (Cussac et al., 2002). Furthermore, a $Cys^{3.35}$Phe mutation in the α_{1b}-AR resulted in constitutive activation of the G_q signalling pathway, but without constitutive activation of G_i signalling, a second pathway activated by this receptor (Perez et al., 1996). Further support for the selectivity of this mutation-induced conformation was provided by the observation that it increased the affinity and potency of one set of structurally similar ligands, the phenethylamines, but had no affect on the affinity or potency of the structurally distinct imidazolines (Perez et al., 1996). Interestingly, different ligands can even facilitate selective interaction of the receptor with different G proteins of the same class, as was observed with the G_i family of G proteins at the CB_1 cannabinoid receptor (Mukhopadhyay and Howlett, 2005).

The signal selectivity of distinct receptor active conformations is not confined to differential G protein signalling. For example, a β_2-AR ligand, propanolol, which acts as

an inverse agonist of G_s signalling at the receptor, paradoxically acts as an agonist in inducing a non-G protein-mediated event involving upregulation of the MAPK pathway (Baker et al., 2003). Additionally, distinct receptor conformations are able to differentially select for signalling and desensitisation events, such as receptor phosphorylation and internalisation. An example includes a bradykinin receptor, which has a mutation within the 7TM domain, that was constitutively phosphorylated and internalised, but did not activate G_q (Kalatskaya et al., 2004). Furthermore, a ligand of the angiotensin AT_{1A} receptor induced desensitisation-associated receptor phosphorylation, but was unable to facilitate receptor internalisation, revealing that distinct conformational states mediate these pathways (Thomas et al., 2000).

1.5.4 LiSS and multiple active conformations of the GnRH receptor

There are two sets of experimental data consistent with the ability of the GnRH receptor to exhibit LiSS. The first involves the observation of reversal of ligand efficacies at distinct GnRH receptor signalling pathways. While GnRH II has a ten-fold lower potency at G_q signalling compared with GnRH I, it exhibits an increased potency in mediating anti-proliferative signalling at the GnRH receptor (Enomoto and Park, 2004; Grundker and Emons, 2003; Millar et al., 2008). Additionally, ligands previously classified as antagonists due to their inability to activate G_q signalling, are reported to induce G_i activation and anti-proliferative signalling at the GnRH receptor (Maudsley et al., 2004; Yano et al., 1994).

Further experimental evidence consistent with LiSS at the GnRH receptor shows that mutations of the receptor can selectively increase the binding affinity and/or potency of the receptor for distinct ligands. This was demonstrated following alanine substitution of $Met^{3.43}$, $Met^{5.54}$, $Phe^{6.40}$, $Phe^{6.44}$ and $Ile^{7.52}$ in TM3, TM5, TM6 and TM7 of the GnRH receptor. Mutation of each of these residues individually, specifically increased the affinities of the mutant receptors for GnRH II, but did not affect the affinities for GnRH I (Lu et al., 2005). As these residues are proposed not to form direct binding contacts with GnRH II, these data suggest that GnRH I and GnRH II preferentially stabilise distinct receptor conformations. Furthermore, mutation of $Asn^{7.45}$ to Ala, which occurs

in the TM domain of the GnRH receptor and is not a direct ligand binding site, increased the affinity and efficacy of GnRH I decapeptides with substitutions of Arg^8 with Gln, Trp or Ser, without significantly altering native GnRH I binding affinity and efficacy (Lu et al., 2007). These results are consistent with the ability of structurally distinct GnRH ligands to bind to and induce different receptor conformations at the GnRH receptor.

1.5.5 Ligand-independent factors that alter receptor conformation

LiSS considers the effects of the extracellular ligand on GPCR structure which induces long-range changes in receptor structure thereby altering the conformation of the intracellular domain of the receptor and thus intracellular signalling. Conversely, GPCR interactions with intracellular proteins, which alter the conformation of the receptor, can change the observed affinity at the extracellular ligand binding pocket (Nelson and Challiss, 2007). Evidence for ligand-independent modulation of receptor conformation is considered in this section.

The earliest evidence that interaction of GPCRs with intracellular proteins altered receptor conformation followed the observation that GPCR affinity was affected by the presence or absence of G proteins (De Lean et al., 1980). High agonist binding affinity was observed at receptors coupled to G proteins whereas uncoupling of the receptor from G proteins by addition of GTP analogues resulted in a low affinity receptor conformation. Other factors that have been observed to influence receptor conformation include phosphorylation of the intracellular domains of the receptor, receptor dimerisation, interactions with accessory proteins (such as receptor activity-modifying proteins (RAMPs) and scaffolding proteins) and localisation of the receptor in the specialised membrane microdomains, namely lipid rafts (Chini and Parenti, 2004; McLatchie et al., 1998; Nelson and Challiss, 2007; Terrillon and Bouvier, 2004). A consequence of the ability of the intracellular milieu to influence receptor conformation is that, as a result of differential expression of proteins in different cells, GPCRs may exhibit differential binding and signalling profiles depending on the tissue in which the receptor is expressed. Other tissue-specific factors that may change the pharmacological

and signalling profile of a receptor in different cells involves differential splicing and editing of the receptor and distinct post-translational modifications (such as palmitoylation and phosphorylation) (McGrew et al., 2004; Nelson and Challiss, 2007).

The GnRH receptor exhibits cell-context dependent signalling (Dobkin-Bekman et al., 2006). Indeed, the large tissue-specific differences in GnRH receptor signalling led researchers to suggest that a second form of the GnRH receptor is expressed by splice variants or otherwise (Enomoto et al., 2004). However, other data argue against the existence of such variants (Grundker et al., 2001). Nevertheless, there is evidence to suggest that the GnRH receptor is subject to a number of the factors discussed above. For example, there have been several reports to indicate that phosphorylation of the GnRH receptor is able to alter ligand binding (Caunt et al., 2004; Liebow et al., 1991). Furthermore, there are several lines of evidence to suggest that the GnRH receptor forms homodimeric complexes (Cheung and Hearn, 2003; Cheung and Hearn, 2005). The GnRH receptor may also interact with a protein, denoted GnRH II reliquum, which is translated from a short mRNA transcript initiated from the cytoplasmic end of TM5 to the carboxyl terminus of the putative type II GnRH receptor gene (Pawson et al., 2005). This protein affects expression of the type I GnRH receptor (Pawson et al., 2005). However, the full effects of this protein on the GnRH receptor function, such as induction of differential signalling, require further elucidation. These data highlight the importance of cellular context when documenting the pharmacological and signalling profile of ligands at a particular receptor.

1.5.6 Revisiting the concept of efficacy and theoretical models for receptor activation

Features of LiSS have necessitated revisiting some of the classical pharmacological terms and theoretical models of receptor activation. One example is the concept of efficacy. Historically, efficacy of a ligand provided a measure of ligand-induced production of downstream second messenger molecules relative to the maximal response observed in the relevant tissue (Kenakin, 2002; Kenakin, 2003). However, the emerging complexity of GPCR behaviour requires the inclusion of responses such as receptor phosphorylation, internalisation, interaction with non-G protein effectors/scaffolds and

activation of multiple G proteins, into the term efficacy (Kenakin, 2002). Within this framework a ligand can demonstrate efficacy at one pathway while having no, or negative efficacy (inverse agonism) at another functional response. This allows for the ability of, for example, antagonists at G protein signalling pathways to have a measure of efficacy at induction of internalisation pathways.

The utility of the ETCM is limited as it cannot explain the observation of reversal of ligand efficacy (Kenakin, 2003). Furthermore, this model considers only the dynamic interactions of the ligand, receptor and a single G protein type (Kenakin, 2004). However, many GPCRs activate multiple G proteins as well as non-G protein interaction partners that induce functional responses at GPCRs. An alternative model to describe receptor behaviour is the probabilistic model (Kenakin, 2004). This model states that in the absence of ligand, instead of existing in two conformations, the inactive and active states, the receptor exists in a number of distinct conformations. Ligand binding, as well as the presence of other factors that bind to the receptor and alter its conformation, change the distribution of receptor conformations increasing the probability of a receptor being in one subset of conformations and decreasing it in others. The subset of receptor conformations stabilised by the ligand determines the set of signalling pathways activated by that receptor (see Fig.1.11). Thus this model can incorporate many different facets of GPCR behaviour and is useful to explain data observed in the complex tissue environments (Kenakin, 2004).

1.5.7 Molecular basis for distinct receptor conformations

Historically, receptors were viewed as switches which can simply be turned on and off. However, the existence of LiSS suggests that the receptor is instead made up of a series of functional domains/molecular switches, each with the capacity to alter the conformation of a specific microdomain within the receptor's structure. Different agonists are able to disrupt a specific subset of these functional domains and this determines the conformation and signalling capacity induced by the ligand (Yao et al., 2006). These functional domains include the highly conserved intramolecular interactions regulating receptor activation (described in section 1.3), specifically the

rotamer toggle switch, the ionic lock and the NPxxY motif (Weinstein, 2005; Yao et al., 2006). However, there are likely to be additional receptor-specific functional domains specialised for the signalling of the relevant GPCR. The molecular mechanisms underlying LiSS are poorly understood and future research efforts should address LiSS on two levels. Firstly, it is important to be able to translate ligand interactions into the disruption of particular functional domains within the receptor structure. Secondly, the disruption of specific permutations of the functional domains/intramolecular interactions needs to be related to the activation of downstream signalling pathways. An example of the coupling of a functional domain to a downstream signalling pathway is the proposal that the NPxxY motif, which makes intramolecular interactions with helix 8, regulates the interaction with and activation of proteins that interact with the C-terminal tail of GPCRs, such as PDZ domain-containing proteins (Weinstein, 2005).

1.5.8 Summary

Understanding the ability of ligands to selectively stabilise distinct receptor conformations and thus direct the activation of the downstream signalling pathways has important therapeutic implications as it presents the possibility that GPCR ligands/drugs can be tailored to induce activation of a desired signalling pathway, but not activation of other signalling pathways that may be associated with side-effects. To attain this level of utility, understanding LiSS requires investigation from several experimental vantage points (Urban et al., 2007). Firstly, it is important to delineate how structurally distinct ligands interact with the receptor to induce different receptor conformations that have selective signalling capacity. Secondly, it is necessary to be able to relate distinct receptor conformations to the specific functional downstream responses activated. Finally, at the most distal level, the role of receptor downstream signalling must be placed in the context of the tissue and organism.

An interest in understanding the molecular mechanisms facilitating LiSS at the GnRH receptor represents the underlying motivation and central theme for the work in this dissertation. In chapter 2, I present an investigation of the ability of Tyr^{5} and His^{5} of GnRH I and GnRH II respectively to interact with $Tyr^{6.58}$ of the GnRH receptor. In view

of the distinct receptor conformations and differential signalling induced by GnRH I and GnRH II, delineation of the similar and contrasting ligand-receptor interaction sites of these two ligands will provide insight into LiSS at the GnRH receptor. In the subsequent chapters, I present investigations into the ability of the GnRH receptor to signal to diverse downstream signalling pathways. In chapter 3, the capacity of the GnRH receptor to interact with multiple G proteins, specifically G_q and G_i, is explored. Subsequently, in chapter 4, a novel G protein-independent signalling pathway involving activation of the SH2 domain-containing phosphatase 2 (SHP-2) by the GnRH receptor is presented. Exploring the functional motifs of the GnRH receptor required to activate downstream signalling pathways will contribute to the understanding of the molecular mechanism whereby the GnRH receptor mediates such diverse physiological effects (see Table 1.1). Ultimately, insight into LiSS at the GnRH receptor may assist in the development of GnRH analogues with increased specificity at target physiological signalling pathways.

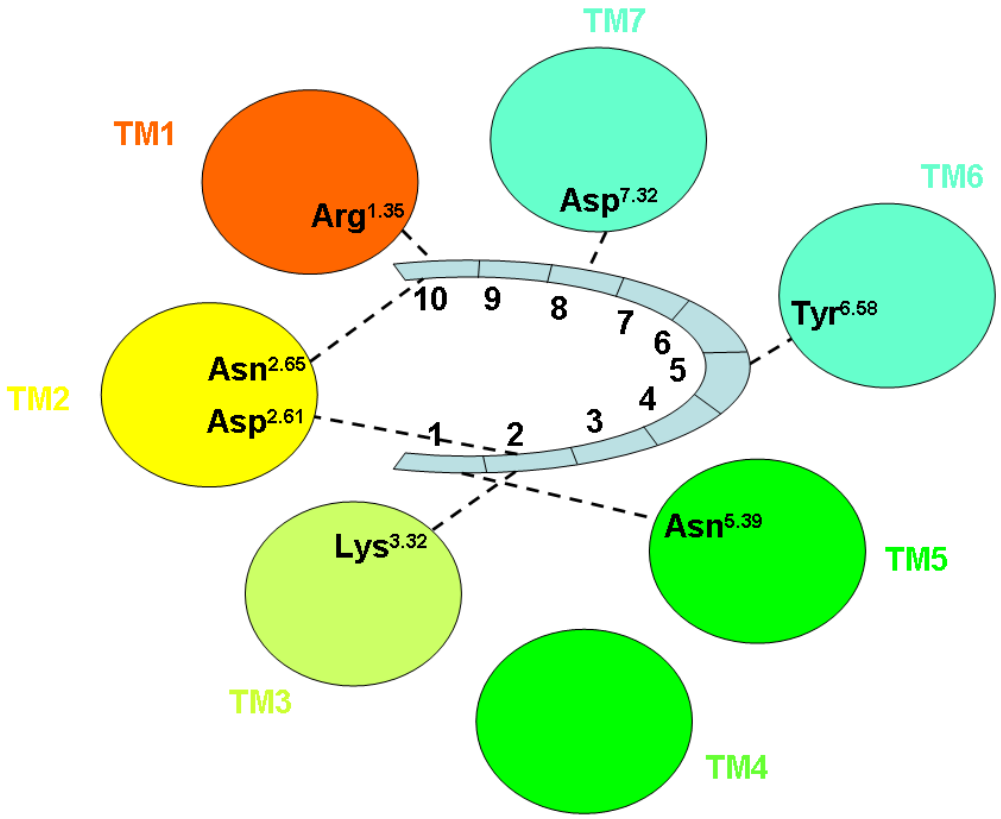
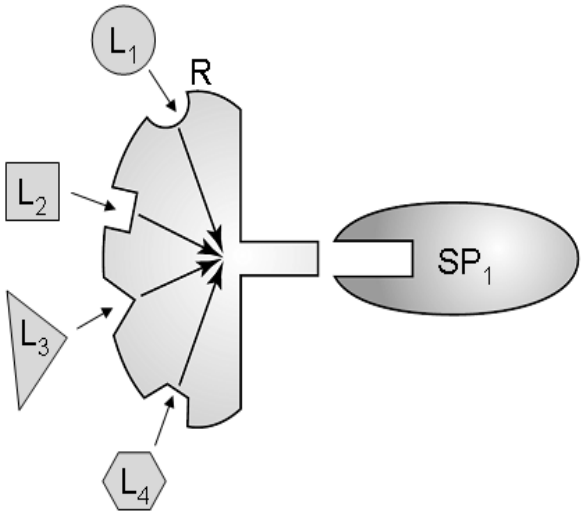


Figure 1.9. Schematic representation of GnRH I binding to the GnRH receptor TM domains. GnRH I is proposed to make the following interactions: $PGlu^{1}$ (1) interacts with $Asn^{5.39}$; His^{2} (2) interacts with $Asp^{2.61}$ and $Lys^{3.32}$; Tyr^{5} (5) interacts with $Tyr^{6.58}$; Arg^{8} (8) interacts with $Asp^{7.32}$; Pro^{9} and the C-terminal glycinamide (10) interact with $Arg^{1.35}$ and $Asn^{2.65}$. This is a schematic representation of Fig.1.6.

A



B

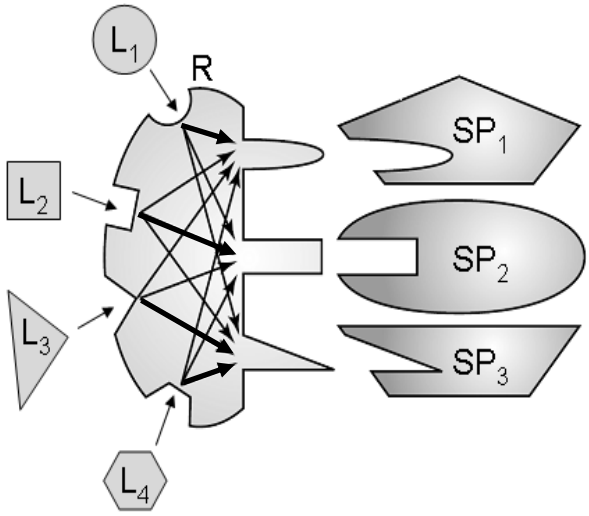


Figure 1.10. The concept of ligand-induced selective signalling (LiSS). A, Historically, GPCRs (R) were viewed as "on-off switches", where many structurally distinct ligands (L_1-L_4) bind to the same receptor type and induce activation of a single downstream signalling pathway (SP_1) by stabilising a single receptor active conformation. B, However, recent evidence suggests that GPCRs can activate multiple downstream pathways (SP_1-SP_3) independently of each other. These signalling pathways may represent multiple G protein signalling pathways, G protein-independent signalling or even initiation of receptor desensitisation and internalisation. LiSS refers to the ability of different ligands to facilitate differential activation of the downstream signalling pathways by stabilisation of distinct active conformations of the receptor, which differ in their capacity to interact with downstream signalling molecules. In the above example, preferential coupling of the different ligands is indicated by larger arrows. Figure is adapted from a previous review (Kenakin, 2003).

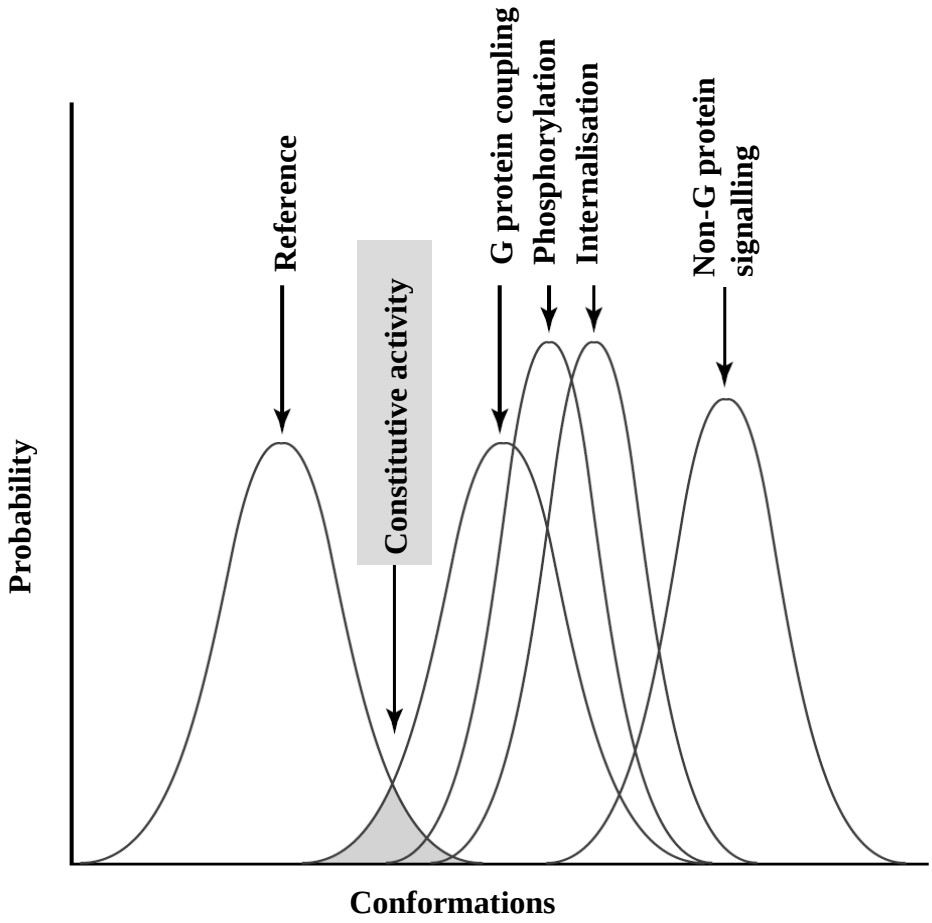


Figure 1.11. Probabilistic theory of GPCR activation. In the probabilistic theory, GPCRs exist in a range of receptor conformations. Each receptor-mediated biological event, such as G protein coupling, receptor phosphorylation, internalisation and non-G protein signalling, is facilitated by a specific ensemble of receptor conformations that may overlap with other receptor-mediated activities. In the resting state, GPCRs occur in a subset of conformations indicated as the reference state that is usually associated with little pharmacological activity. However, a small percentage of the receptor conformations in the reference state may overlap with, for example, the G protein coupling conformation, thus giving rise to constitutive activity (grey shaded area) as indicated in the example above. Ligand binding or accessory proteins alter the distribution of receptor conformations thereby changing the biological activities induced by the receptors. Figure adapted from a previous review (Kenakin, 2002).

1.6 GnRH receptor signalling

GnRH receptor signalling has been studied extensively and is the subject of several reviews (Caunt et al., 2006; Dobkin-Bekman et al., 2006; Kraus et al., 2001; Kraus et al., 2006; Naor et al., 2000; Naor et al., 1998; Pawson and McNeilly, 2005). However, a comprehensive account of these signalling pathways is beyond the scope of this review. Instead, I focus on two signalling pathways investigated in this thesis, which are suggested to facilitate the anti-proliferative and proapoptopic signalling of GnRH receptor, specifically coupling of the receptor to the alternative G protein family, G_i, and activation of protein tyrosine phosphatase (PTP) signalling (Imai et al., 1996a; Maudsley et al., 2004). Thus, in the final section of this review, multiple G protein signalling and activation of the SH2-domain containing phosphatases (SHPs) by GPCRs are discussed.

1.6.1 Activation of multiple G proteins by the GnRH receptor is proposed to enable signalling to distinct downstream pathways

Historically, GPCRs were thought to mediate activation of downstream signalling pathways by coupling exclusively to one G protein class. However, recent evidence has revealed that a single GPCR can couple to multiple G protein classes with distinct downstream signalling effects (Hermans, 2003; Kukkonen, 2004). Furthermore, as discussed above, structurally distinct ligands can induce different conformations at the same receptor, which differ in their ability to activate different classes of G proteins (Akam et al., 2001; Gazi et al., 2003; Perez and Karnik, 2005; Reversi et al., 2005; Urban et al., 2007). Thus some ligands may be able to activate a single G protein class, while others may activate two G protein families with differing efficacies.

The GnRH receptor is able to induce anti-proliferative and proapoptopic signalling in reproductive cancer cells (Grundker and Emons, 2003; Grundker et al., 2001; Kraus et al., 2006; Maiti et al., 2005; Maudsley et al., 2004). However, activation of this pathway does not correlate with classical GnRH receptor signalling in the pituitary, which involves activation of the $G_{q/11}$ family of G proteins (Grundker and Emons, 2003; Millar et al., 2008). This has led to the proposal that the anti-proliferative effects of the GnRH receptor are mediated by activation of a distinct G protein family (Millar et al., 2004;

Millar et al., 2008; Stanislaus et al., 1998b). Indeed, there is evidence to suggest that the G_i family of G proteins is involved in GnRH receptor anti-proliferative signalling (Grundker et al., 2001; Imai et al., 2006; Limonta et al., 1999). However, the ability of the GnRH receptor to couple directly to G_i is still a matter of debate (Grosse et al., 2000). In this section, GPCR-G protein coupling is critically discussed with emphasis on data specific to GnRH receptor-G protein coupling. As the GnRH receptor is also proposed to couple to G_s, evidence to support this interaction is also presented.

1.6.1.1 Determination of GPCR-G protein coupling preferences

Determination and classification of the G protein classes activated by a GPCR are often inferred from the second messenger systems activated by the receptor, which are specific to that G protein type (see Table 1.2). Activation of G_q results in activation of phospholipase Cβ (PLCβ), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2), thereby generating the second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). In contrast, G_s and G_i have opposing effects on adenylate cyclase (AC), where G_s is stimulatory and G_i is inhibitory, resulting in respective generation or inhibition of the second messenger cAMP. Thus if GPCR activation results in generation of IP_3, accumulation of cAMP or reduction in cAMP production, the GPCR is frequently classified as a G_q-, G_s- or G_i-coupled receptor respectively. However, the effectors, PLCβ and adenylate cyclase, can be indirectly regulated by alternative G protein classes. For example, Gβγ subunits dissociated from activated Gα subunits, such as G_s and G_i, are able to activate PLCβ (Table 1.3). Additionally, Ca^{2+} ions are able to differentially activate or inhibit certain isoforms of AC (Birnbaumer, 2007). Ca^{2+} ions released from intracellular stores following IP_3 generation may result in effects on AC in response to a G_q-coupled GPCR. Thus designation of the G protein class activated by a GPCR, based on analysis of the observed regulation of second messenger systems, can result in erroneous conclusions.

Another mechanism used to infer GPCR-mediated G protein activation involves the use of toxins that specifically activate or inhibit the relevant G protein class. For example,

cholera toxin (CTX) facilitates ADP-ribosylation of G_s proteins inducing the constitutive activation of G_s. If CTX is able to induce a similar cellular response as that of the GPCR, some researchers conclude that the GPCR is able to activate G_s. However, as discussed above, this may not be accurate as other G protein subtypes may mediate an increase in cAMP. Another toxin, pertussis toxin (PTX) induces ADP-ribosylation and inactivation of the G_i family of G proteins and can thus be used to suggest activation of this G protein subtype in response to a GPCR (Cabrera-Vera et al., 2003). However, the ability of GPCRs to transactivate RTKs, which can activate G proteins, suggests that use of this toxin is also not able to prove conclusively that a specific G protein class is coupled to a GPCR (Kreuzer et al., 2004; Shan et al., 2006a).

The use of second messengers and toxins to determine the G protein coupling preference of a GPCR is therefore problematic. Thus in order to conclusively deduce the G protein activated by a GPCR, the most proximal step of the receptor-stimulated G protein activation pathway should be assessed. The activation of a G protein by a GPCR involves the exchange of a GDP molecule for GTP on the G protein (see section 1.2). Arguably one of the best methods to a capture this activation event is the [^{35}S]GTPγS binding assay, which uses a radioactive non-hydrolysable GTP analogue to label activated G proteins (Harrison and Traynor, 2003; Lazareno, 1999; Milligan, 2003; Weiland and Jakobs, 1994). This method is performed in membranes eliminating cross-talk that would be observed in the intact cell and can quantitatively measure activation of a specific G protein class.

1.6.1.2 The GnRH receptor is proposed to couple to multiple classes of heterotrimeric G proteins

Numerous studies suggest that the GnRH receptor couples to multiple G protein classes, including G_q, G_s and G_i. Table 1.4 provides a list of the G proteins that the GnRH receptor is proposed to activate, the experimental method used to suggest which G protein class is involved and the downstream signalling affected by activation/inhibition of the relevant G protein. The majority of these studies rely on second messenger systems and pertussis and cholera toxins to infer the involvement of the relevant G

proteins. Only two studies measured either a direct GnRH receptor-G protein interaction or receptor-stimulated exchange of GDP for GTP (see Table 1.4). The first study showed cross-linking of G_q and G_i to the GnRH receptor (Grundker et al., 2001). However, this interaction was only investigated in the absence of agonist. Thus the specificity of the interaction and the ability of the GnRH receptor to activate the G proteins (which would be expected to induce receptor-G protein dissociation and thus decrease the quantity of G proteins cross-linked to the receptor) was not assessed. The second study captured GnRH receptor-catalysed G protein activation by measuring the binding of a non-hydrolysable GTP analogue, [α-^{32}P]GTP azidoanilide, to the G proteins in the presence or absence of agonist (Grosse et al., 2000). The assay was performed in membranes which eliminated the possibility of signal cross-talk. Investigation of GnRH receptor-induced photolabelling of G_q, G_s, G_i, G_{12} and G_{13} with [α-^{32}P]GTP azidoanilide revealed that only G_q was activated in response to GnRH I (Grosse et al., 2000). However, this contrasts with other, albeit less direct methods, that suggest that G_s and G_i specifically, rather than their downstream second messengers, are indeed involved in GnRH receptor signalling. For example, G_s and G_i proteins undergo agonist-stimulated palmitoylation and release from the plasma membrane following GnRH receptor activation (Krsmanovic et al., 2003; Stanislaus et al., 1998c). Furthermore, the ability of GnRH receptor agonists to antagonise PTX-catalysed ADP ribosylation of G_i in a dose-dependent manner suggests that G_i is activated downstream of the GnRH receptor (Grundker et al., 2001; Limonta et al., 1999). Thus, the ability of the GnRH receptor to couple to G_s and G_i is still a matter of debate.

1.6.1.3 Receptor determinants of receptor-G protein coupling

Due to the low sequence homology of the ICLs of GPCRs, efforts to designate the G protein coupling preference of a receptor based on analysis of the primary sequence have proved largely unsuccessful (Wess, 1998). Instead, similar to the paradigm in the 7TM domain, the ICLs of GPCRs are proposed to share a similar conformation, rather than sequence homology, that facilitates G protein recognition and activation (Wong, 2003). Supported by the observed activation of G proteins by short peptide sequences, the

receptor conformation which facilitates G protein activation is suggested to be an amphiphilic α-helical structure with hydrophobic and cationic residues on opposing sides of the helix (Wong, 2003). This structure is suggested to activate all G protein classes, including $G_{i/o}$, G_s and G_q proteins (Wong, 2003). This has led to the hypothesis that ICLs constitute two functional domains, an activation domain that interacts with and activates the G protein and a selectivity domain that restricts access to the activation domain to a specific G protein subtype (Wong, 2003).

Analysis of the functional domains of the ICLs of GPCRs that are important for G protein activation have revealed the importance of ICL2 and the N- and C-terminal regions of ICL3 (Wess, 1998). This is supported by ICL-swapping experiments that enabled recipient GPCRs to activate the G protein class of the donor receptor (Wess, 1998). In contrast, consensus interpretation of the roles of ICL1 and the C-terminal tail suggest that they are predominantly involved in regulating the selectivity and efficiency of the receptor-G protein interaction (Wess, 1998). However, the importance of these domains varies among different GPCRs (Wess, 1998).

In the GnRH receptor, residues in ICL1, ICL2 and ICL3 are proposed to mediate receptor-G protein specificity. ICL1 of the GnRH receptor exhibits two basic residues at its N-terminal end and a BBxxB (where B is a basic amino acid and x is any residue) motif at the C-terminal end of the loop (see Fig.1.8). This motif is suggested to facilitate G_s and G_i coupling in other ICLs of the GPCR family (Arora et al., 1998). Site-directed mutagenesis of residues within ICL1 of the GnRH receptor severely compromised cAMP accumulation, but did not affect IP signalling in COS-7 cells (Arora et al., 1998). This suggests that the cAMP and IP signalling pathways can be activated independently of each other and that ICL1 may facilitate selective coupling to G_s proteins. Mutation of residues in ICL2 has revealed the importance of this loop in IP signalling and thereby suggested a role for ICL2 in G_q coupling (Arora et al., 1997; Arora et al., 1995; Ballesteros et al., 1998). Furthermore, other experimental data suggest that ICL3 is also important for G_q and G_s coupling. For example, mutation of $Ala^{6.29}$ (at the C-terminal end of ICL3) to amino acids with larger side chains abolished IP signalling, suggesting

that ICL3 facilitates G_q coupling (Myburgh et al., 1998a). Furthermore, overexpression of GnRH receptor ICL3 peptides in GGH_3 cells resulted in inhibition of GnRH receptor-induced IP signalling and cAMP production, suggesting that this loop facilitates both G_s and G_q coupling (Ulloa-Aguirre et al., 1998). However, the ability of ICL3 of the G_q-coupled M_1 muscarinic receptor to mediate inhibition of GnRH-induced cAMP accumulation questions the specificity of this experimental approach (Ulloa-Aguirre et al., 1998). In summary, ICL1 and ICL3 are proposed to facilitate G_s coupling, while ICL2 and ICL3 are suggested to enable G_q coupling by the GnRH receptor. To date, no domains have been implicated in GnRH receptor activation of G_i.

1.6.1.4 Other determinants of receptor-G protein coupling

In addition to selectivity imposed by the sequence of the GPCR, there are a number of other factors that regulate the selectivity of the receptor-G protein interaction (Albert and Robillard, 2002; Wess, 1998). The most obvious of these are the determinants inherent in the G protein sequence. The C-terminal amino acids of the Gα protein are critically involved in receptor selectivity (Oldham and Hamm, 2006; Slessareva et al., 2003; Wess, 1998). Furthermore, other regions of Gα, such as the N-terminus, the α4/β6 loop, the αN/β1 loop, the α2/β4 loop and the α3/β5 loop are also suggested to regulate interaction with the receptor, but may be specific to the G protein class involved (Oldham and Hamm, 2006; Slessareva et al., 2003). In addition to Gα, the subtype composition of the Gβγ subunit is also proposed to modulate receptor-G protein selectivity (Albert and Robillard, 2002; Oldham and Hamm, 2006). Other factors that affect receptor-G protein coupling specificity include G protein expression levels, modifications of the receptor (such as phosphorylation), interactions of RGS proteins with the G protein and interactions of scaffolding proteins with the receptor (Albert and Robillard, 2002). Thus the G protein selectivity observed at a particular GPCR may be influenced by the cell type in which it is expressed.

1.6.1.5 Summary

The GnRH receptor is suggested to exhibit promiscuous coupling to G_q, G_s and G_i (Table 1.4). However, the use of second messenger systems to designate these coupling preferences and conflicting reports which suggest that the GnRH receptor only couples to G_q (Grosse et al., 2000), highlight the necessity to investigate GnRH receptor coupling using techniques that directly measure receptor-stimulated G protein activation. Furthermore, the influence of ligand structure on these distinct signalling pathways and the receptor domains required for preferential G protein coupling are not well-defined. The importance of investigating these pathways is underscored by proposals suggesting that the activation of multiple G proteins may facilitate the diverse physiological effects of the GnRH receptor (Millar et al., 2008; Stanislaus et al., 1998b). As I am interested in the anti-proliferative and proapoptopic effects of the GnRH receptor in reproductive cancers, I chose to focus on G_i activation, as this G protein is proposed to mediate these effects (Limonta et al., 1999; Maudsley et al., 2004). Additionally, the molecular mechanisms underlying the proposed interaction between the GnRH receptor and G_i are poorly understood. To address this, in chapter 3, I set up the [^{35}S]GTPγS binding assay to measure the ability of the GnRH receptor to directly activate both G_q and G_i.

Table 1.4. Investigation of multiple G protein coupling of the GnRH receptor

G protein(s)	Cell type	Method used to infer G protein involvement	Direct method	Reference
G_i	αT3	PTX-sensitive MAPK activation.	No	(Sim et al., 1995)
G_i	Human reproductive tumours	Dose-dependent protection of G_i from ADP-ribosylation by PTX in the presence of increasing concentrations of a GnRH agonist. PTX inhibits PTP activity.	No	(Imai et al., 1996b)
G_i	LNCaP and DU145 prostate cancer cells	Ability to decrease forskolin-stimulated cAMP responses. GnRH agonist antagonises PTX-catalysed ADP ribosylation of G_i. PTX inhibits the antiproliferative effects.	No	(Limonta et al., 1999)
G_i	Ovarian cancer cell line	PTX inhibits ERK activation.	No	(Kimura et al., 1999)
G_i	Prostate cancer JEG-3 and BPH-1 cells	Inhibition of cAMP.	No	(Maudsley et al., 2004)
G_i, G_q	Ishikawa, Hec-1A endometrial cancer cells and EFO-21 and EFO-27 ovarian cancer cells	Cross-linking experiments indicate GnRH receptor coupling to G_q and G_i. PTP activity and EGF-induced c-fos expression are PTX-sensitive. GnRH agonist antagonises PTX-catalysed ADP ribosylation of G_i.	Yes	(Grundker et al., 2001)
G_i, G_s	FNC-B4 neurons	cAMP accumulation, which is PTX-sensitive at high agonist concentrations. Migration is PTX-sensitive.	No	(Romanelli et al., 2004)
G_i, G_s	Gonadotrope	PTX-sensitive IP production, CTX-enhanced LH release. CTX and PTX decreased GnRHR binding.	No	(Hawes et al., 1993; Hawes and Conn, 1993; Hawes et al., 1992)
G_i, not G_s	Ovarian cancer cells	PTX inhibits induction of apoptosis and PP2A redistribution. CTX does not affect this pathway.	No	(Imai et al., 2006)
G_q	αT3	IP_3 and DAG production	No	(Poulin et al., 1996)
G_q	αT3	$G_{q/11}$ antibody inhibits PLC activity	No	(Hsieh and Martin, 1992)
G_q	*In vivo* G_q and G_{11} knockout mice	LH and steroid (testosterone and estradiol) production affected, but not abolished in mouse knockouts of either G_q or G_{11}.	No	(Stanislaus et al., 1998a)
G_q	Pituitary cells	Laser-scanning microscopy and immunohistochemistry reveal redistribution of $G_{q/11}$ in response to a GnRHR agonist	No	(Cornea et al., 1998)

G protein(s)	Cell type	Method used to infer G protein involvement	Direct method	Reference
G_q	Purified gonadotrope	Phosphoinositide hydrolysis and DAG formation.	No	(Andrews and Conn, 1986)
G_q	Rat pituitary cell cultures, GGH_3	Palmitoylation labelling of the $G_{q/11}$. IP production. Downregulated expression of $G_{q/11}$ in response to a GnRH agonist.	No	(Stanislaus et al., 1997)
G_q, G_s	COS-7	cAMP and IP production.	No	(Arora et al., 1998)
G_q, G_s	CV-1 cells	IP and cAMP production.	No	(Oh et al., 2003)
G_q, G_s	GGH_3	GnRHR third intracellular loop peptide competitively inhibited IP and cAMP production and PRL release.	No	(Ulloa-Aguirre et al., 1998)
G_q, G_s	GGH_3	IP production, cAMP accumulation.	No	(Awara et al., 1996)
G_q, G_s	Sf9 insect cells	IP and cAMP production.	No	(Delahaye et al., 1997)
G_q, G_s, G_i	GGH_3	IP production, CTX and PTX inhibit PRL production and augment IP production.	No	(Janovick and Conn, 1994)
G_q, G_s, G_i	Gonadotrope	PTX inhibits IP production. CTX decreased IP production and enhanced LH release.	No	(Barnes and Conn, 1993)
G_q, G_s, G_i	GT1-7 neurons	Measurement of cAMP (stimulation at low agonist concentrations and inhibition at high agonist concentrations), Ca^{2+} and IP production, and release of membrane-bound Gα subunits for all G protein types.	No	(Krsmanovic et al., 2003)
G_q, G_s, G_i, G_{14} and G_{15}	Rat pituitary cells, GGH_3	Receptor-stimulated palmitoylation of G_i and G_s protein types. Enhanced or decreased cAMP production when G_s or G_i are overexpressed. IP production enhanced by G_q, G_{11}, G_{14} and G_{15} overexpression.	No	(Stanislaus et al., 1998c)
G_q, G_s, not G_i	GGH_3	G_q and G_s involvement in MAPK signalling suggested by PKC- and PKA-dependence of this pathway. PTX has no effect on MAPK signalling.	No	(Han and Conn, 1999)
G_q, G_s, not G_i	LβT2	ERK, c-fos and LHβ induction are PTX-insensitive, but affected by interference with G_q and G_s action. G_q and G_s protected from trypsin digestion in presence of GnRH agonist and GTP analogue.	No	(Liu et al., 2002)
G_q, not G_i	αT3	MAPK activation is not sensitive to PTX, thus suggest G_q.	No	(Reiss et al., 1997)
G_q, not G_i	αT3	IP production which was PTX-insensitive.	No	(Anderson et al., 1993)
G_q, not G_i, G_s or $G_{12/13}$	αT3, CHO-K1, COS-7	IP production and G_q protein GTP photolabelling. Lack of photolabelling of all other G protein types. IP, Ca^{2+} release and ERK signalling are PTX-insensitive. No cAMP or cAMP response element responses.	Yes	(Grosse et al., 2000)

G protein(s)	**Cell type**	**Method used to infer G protein involvement**	**Direct method**	**Reference**
G_q, not G_s or G_{12}	αT3	Time and dose-dependent downregulation of G proteins G_q and G_{11} in response to a GnRH agonist (assessed by immunoblot). G_s and G_{12} levels were not affected.	No	(Shah and Milligan, 1994)
G_s	GGH_3	PRL release in a cAMP-dependent manner.	No	(Stanislaus et al., 1996)
G_s	GGH_3	PRL release facilitated by cAMP production, which is mimicked by CTX.	No	(Kuphal et al., 1994)
G_s	Ovarian tumour cells	cAMP production. cAMP analogues induce ERK activation.	No	(Chamson-Reig et al., 2003)
G_s, not G_i	GGH_3	cAMP production. cAMP analogues and CTX enhance GnRHR gene transcription. AC inhibitor decreased transcription. PTX did not have an effect.	No	(Lin and Conn, 1998)
G_s, not G_i	Rat pituitary	CTX enhances and sensitises the LH release response. LH release is not affected by PTX.	No	(Janovick and Conn, 1993)
Not G_s	Primary rat culture pituitary cells	No effect of CTX or cAMP potentiating agents or analogues on LH release.	No	(Conn et al., 1979)

Some of the references in this table were obtained from a previous review (Ruf et al., 2003).

1.6.2 GPCR-mediated G protein-independent signalling and regulation of phosphotyrosine signalling

Regulation of phosphotyrosine signalling is an important signal transduction mechanism that regulates a wide variety of essential physiological processes, including proliferation, migration, adhesion and differentiation (Ostman et al., 2006; Stoker, 2005). The phosphotyrosine content of a cell is tightly regulated by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). This equilibrium is frequently dysregulated in cancer cells. Consistent with this, PTKs are able to transform cells while PTPs often exhibit tumour suppressor activity (Ostman et al., 2006).

Historically, receptor-activated phosphotyrosine signalling was considered to be exclusively mediated by receptor tyrosine kinases (RTKs) and receptor tyrosine phosphatases (RTPs). However, emerging data indicates that GPCRs also regulate tyrosine phosphorylation cascades (Gavi et al., 2006; Lowes et al., 2002; Luttrell and Luttrell, 2004). Specifically, GPCRs have been shown to initiate transactivation of receptor tyrosine kinases (RTKs), activation of the cytoplasmic tyrosine kinase, src, and activation of PTPs (Florio, 2008; Luttrell and Luttrell, 2004). Interestingly, while some GPCRs activate these pathways downstream of G protein signalling, recent experimental evidence suggests that certain GPCRs are able to directly interact with and activate PTKs/PTPs, independently of G proteins (Florio, 2008; Sun et al., 2007b). The ability of GPCRs to interact with and/or activate PTK/PTP molecules, independently of G proteins, presents the possibility that the structural requirements for activation of the two distinct pathways may differ. Thus investigation of the molecular determinants which facilitate activation of the PTK/PTP pathway and the differential capacity of different ligands to activate this pathway, when compared to G protein signalling, may facilitate the understanding of LiSS at the GPCR. In this review, I will focus on the molecular mechanisms that are proposed to facilitate GPCR direct interactions with two types of the PTKs/PTPs, specifically src and the SH2 domain-containing phosphatases (SHPs).

1.6.2.1 GPCRs can bind directly to src

Src is a member of the src family of protein tyrosine kinases (SFKs). These proteins play important roles in the regulation of cell growth, differentiation, cell shape, migration and survival (Parsons and Parsons, 2004). Consistent with this, src activity is frequently upregulated in many cancers and is considered an oncogene (Parsons and Parsons, 2004). SFKs are critical and well-established downstream signalling partners of growth factor receptors/RTKs (Bromann et al., 2004). However, src activation by GPCRs is now also well-established and is proposed to facilitate the effects of GPCRs on cell proliferation, cytoskeleton remodelling and in GPCR trafficking (Luttrell and Luttrell, 2004).

Src consists of an N-terminal domain, followed by an SH3, an SH2, a kinase domain and a short C-terminal tail (Boggon and Eck, 2004). Inactive src exists in an autoinhibited conformation where the SH3 and SH2 domains fold back against the kinase domain making intramolecular interactions that lock the kinase in an inactive conformation. An interaction that stabilises this conformation is binding of the SH2 domain to an autoinhibitory phosphorylated tyrosine residue in the C-terminal tail (Boggon and Eck, 2004). Src activation is initiated by engagement of its SH2 and/or SH3 domains with appropriate motifs or by dephosphorylation of the autoinhibitory tyrosine residue in its C-terminal tail. These events enable autophosphorylation of a tyrosine residue within the kinase domain and activation of src (Boggon and Eck, 2004).

There are a number of ways in which GPCRs can form direct complexes with src. One mechanism of GPCR-src interaction involves utilisation of β-arrestin as a scaffold. The ability of GPCRs to form a complex with β-arrestin and src, thereby facilitating MAPK activation, has been suggested for several GPCRs, including the β_2-adrenergic and angiotensin (AT_{1A}) receptors (Luttrell et al., 1999; Sun et al., 2007b; Wei et al., 2003). A second mechanism for GPCR-src interactions involves GPCR engagement of the src SH2 domain (Fan et al., 2001). The β_2-AR is suggested to recruit src by a canonical SH2-binding site, pYxxL (where pY is a phosphorylated tyrosine and x is any residue),

in its C-terminal tail (Fan et al., 2001). This interaction enables activation of src and is believed to facilitate GPCR desensitisation and internalisation, in addition to the activation of MAPK cascades (Fan et al., 2001). GPCRs are also able to interact directly with src SH3 domains. Indeed, the serotonin 5-HT_6 receptor C-terminal tail binds to the SH3 domain of a SFK member, fyn, thereby initiating MAPK signalling (Yun et al., 2007). Furthermore, the β_3-AR exhibits SH3-binding proline rich (PxxP) motifs in its third intracellular loop and C-terminal tail, which are proposed to directly interact with src (Cao et al., 2000). Finally, a splice variant of the CCK2 receptor is able to bind to and directly activate src via a 69 amino acid insertion in its third intracellular loop by an, as yet, undefined mechanism (Olszewska-Pazdrak et al., 2004).

Thus numerous GPCRs are able to bind to src, either using β-arrestin as a scaffold or by direct engagement of the SH2 or SH3 domains of src, thereby facilitating src activation. These mechanisms of src activation can occur in a G protein-independent manner (Sun et al., 2007b). For example, an angiotensin II analogue is able to recruit β-arrestin and initiate MAPK activation without activation of G protein signalling (Wei et al., 2003). This implies that β-arrestin recruitment and G protein signalling can occur independently of each other, suggesting that src activation via interaction with the GPCR-β-arrestin complex could also occur independently of G protein signalling. Additionally, expression of a peptide of the third intracellular domain of a CCK2 splice variant was able to bind to and activate src, suggesting that the mechanism of src activation by this receptor is G protein-independent (Olszewska-Pazdrak et al., 2004). Sun et al. have provided convincing evidence that the β_2-AR is also able to activate src independently of G proteins (Sun et al., 2007a). They show that that the β_2-AR can initiate src-dependent MAPK activation in $G\alpha_s$ knockout cells in the presence of PTX, eliminating the possibility that G_s and G_i are involved in this pathway (Sun et al., 2007a). Furthermore, it was shown that the β_2-AR can activate src in a purified *in vitro* system in the absence of G proteins (Sun et al., 2007a). The molecular mechanisms for src activation by this receptor appear to be cell type specific, as utilisation of β-arrestin, an SH2 binding site and a motif in helix 8 of the C-terminal tail have all been suggested

to mediate src activation by the β_2-AR in different cell types (Fan et al., 2001; Luttrell et al., 1999; Sun et al., 2007a).

The GnRH receptor is also able to activate src in many different cell systems, utilising varied mechanisms, including transactivation of the epidermal growth factor receptor (EGFR) or via activation downstream of PKC, depending on the cell context (Dobkin-Bekman et al., 2006; Kraus et al., 2001; Kraus et al., 2006; Naor et al., 2000). Notably, in αT3 cells, a PKC inhibitor was only partially effective in inhibiting src activation leading to the suggestion that src activation by the GnRH receptor has a G_q-independent component (Benard et al., 2001). A Gβγ scavenger and β-arrestin dominant negative construct were unable to reduce GnRH-induced ERK activation, suggesting that these proteins do not contribute to src activation by the GnRH receptor in αT3 cells (Benard et al., 2001). This raises the possibility that the GnRH receptor may facilitate direct activation of src. Indeed, in chapter 4, I show that the GnRH receptor forms a complex with src, as detected by immunoprecipitation experiments, and propose a mechanism to explain how this interaction occurs.

1.6.2.2 GPCRs can bind directly to SHPs

In addition to direct interactions with src PTKs, GPCRs form direct interactions with a family of cytoplasmic PTPs, the SHPs. Historically, SHPs were identified as signalling partners of immunoreceptors, binding to these receptors via immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and thereby facilitating the negative regulatory signals of these receptors (Barrow and Trowsdale, 2006; Coggeshall et al., 2002; Underhill and Goodridge, 2007; Unkeless and Jin, 1997). Early identification of SHP-binding ITIMs suggested that the consensus sequence was V/IxpYxxL/V (where pY is a phosphorylated tyrosine residue and x is any amino acid) (Unkeless and Jin, 1997). However, subsequent studies have suggested a much broader motif specificity, particularly at the -2 position relative to the essential phosphorylated tyrosine residue of the motif (Imhof et al., 2006; Sweeney et al., 2005; Wavreille et al., 2007). This broad specificity underlies the ability of SHPs to interact with many receptors other than the cytokine

receptors, including growth factor receptors and GPCRs (Florio, 2008; Wang et al., 2006).

SHPs are involved in the regulation of many cell processes, including differentiation, cell proliferation, survival and apoptosis, cytoskeletal maintenance and chemotaxis (Chong and Maiese, 2007; Stein-Gerlach et al., 1998). They mediate these effects by influencing a number of signalling pathways, including the PI3K/Akt, JAK/STAT and MAPK cascades (such as ERK and JNK) and NF-κB (Chong and Maiese, 2007). SHP-1 is mainly involved in negative regulation of these pathways (Chong and Maiese, 2007). In contrast, SHP-2 signalling can be either positive or negative depending on the cell context (Chong and Maiese, 2007). Furthermore, SHP-1 is predominantly expressed in haematopoietic cells, while SHP-2 is ubiquitously expressed (Chong and Maiese, 2007; Neel et al., 2003).

The basic structure of the SHPs consists of two SH2 domains, denoted the N-terminal SH2 (N-SH2) and C-terminal SH2 (C-SH2) domains, which are followed by the phosphatase domain and a C-terminal tail which contains two tyrosine phosphorylation sites (Neel et al., 2003; Poole and Jones, 2005). Reminiscent of src, in the inactive state, the N-SH2 domain of SHP is folded over and engages with the catalytic cleft of the phosphatase domain. The C-SH2 domain is unaffected by this interaction and is proposed to function as sensor for phosphorylated docking sites for the protein (Neel et al., 2003). There are two proposed mechanisms for activation of the SHPs (Neel et al., 2003). The first mechanism involves engaging the N- and C-SH2 domains with appropriately phosphorylated motifs. The second mechanism requires phosphorylation of the two tyrosine residues pY536/pY542 and pY564/pY580 in the C-terminal tail of SHP-1/SHP-2 (Neel et al., 2003). These residues bind to the N-SH2 and C-SH2 domains respectively, relieving inhibition of the phosphatase and facilitating its activation.

SHPs are activated downstream of a number of GPCRs, including the chemokine CXCR4 (Chernock et al., 2001; Fernandis et al., 2004; Hoff and Brunner-Weinzierl, 2007), chemokine CCR5 (Ganju et al., 2000), chemokine CCK2 (Vatinel et al., 2006),

type A endothelin-1 (Bisotto and Fixman, 2001), angiotensin AT_1 (Fernstrom et al., 2005; Godeny et al., 2007; Marrero et al., 1998), somatostatin (Florio, 2008) and bradykinin B_2 receptors (Duchene et al., 2002). Within these receptor systems, SHPs have been implicated in the regulation of migration, chemotaxis, cytoskeletal remodelling, proliferation and apoptosis. Interestingly, some GPCRs have been shown to bind directly to SHPs by exhibiting ITIM-like sequences within ICL3 or its TM7/C-terminal tail, which enable binding of SHP SH2 domains. Using co-immunoprecipitation and surface plasmon resonance experiments, direct interactions have been shown between the sst2 somatostatin receptor and SHP-2 (Ferjoux et al., 2003), the bradykinin B_2 receptor and SHP-2 (Duchene et al., 2002) and the CCK2 receptor and SHP-2 (Vatinel et al., 2006). Additionally, glutathione s-transferase (GST) pulldown assays suggest that an ITIM-like (YIPP) motif in the angiotensin AT_1 receptor facilitates SHP-2 binding (Marrero et al., 1998). Furthermore, co-immunoprecipitation experiments suggest that the sst2 somatostatin receptor and SHP-1 form a complex (Lopez et al., 1997). These interactions were necessary for agonist-induced activation of the relevant SHPs by the GPCRs.

It is difficult to extract a common mechanism of GPCR-induced SHP activation from the literature due to the paucity of existing data and because, analogous to src activation, the mechanism of SHP activation is often receptor and cell-context dependent. The GPCRs for which the most experimental evidence for receptor-induced SHP activation exists, are the somatostatin receptors (Florio, 2008). In this receptor system, SHP activation requires src/PTK activity, which in turn requires G_i activation (Florio, 2008), probably because the dissociated Gβγ subunits activate src activity (Ferjoux et al., 2003). Thus the mechanism of src activation (G protein dependent or independent) may dictate the mechanism of SHP activation and is expected to differ for different GPCRs.

Activation of the GnRH receptor has been shown to induce PTP activity (Grundker et al., 2001; Imai et al., 1996a; Imai et al., 1996b). It is also known to antagonise the phosphorylation and signalling of two growth factor receptors, the EGFR and the insulin-like growth factor type I receptor (IGF-IR) in certain cell types (Marelli et al.,

1999; Moretti et al., 1996; Rose et al., 2004). These effects are proposed to enable the anti-proliferative effects of this receptor (Imai et al., 1996a). However, activation of SHP-1 and SHP-2 by the GnRH receptor has not been evaluated. Considering the anti-proliferative activity of the GnRH receptor and its ability to upregulate PTP activity, in chapter 4, I investigate GnRH receptor activation of the SHPs and whether this activation can be mediated by direct interactions with the receptor, independently of G proteins.

1.6.2.3 Physiological relevance of G protein-independent signalling

A key issue is whether GPCR-mediated G protein-independent signalling has physiological relevance or whether this phenomenon is simply a result of the overexpression of the receptors in heterologous recombination systems. This question was addressed by a group which created transgenic mice specifically expressing a wildtype or mutant angiotensin AT_1 receptor in the heart (Zhai et al., 2005). The mutant AT_1 receptor (denoted AT_1-i2m) exhibited an altered second intracellular loop that prevented activation of the downstream G proteins, G_q and G_i, but which did not interfere with G protein-independent signalling, involving activation of src and ERK (Zhai et al., 2005). This study revealed that the mutant receptor was able to induce cardiac hypertrophy and other alterations in cardiac function, suggesting that certain effects of the receptor can be mediated by G protein-independent mechanisms *in vivo* (Zhai et al., 2005).

Thus, assuming that G protein-independent signalling has physiological significance, what are the determining factors for induction of G protein-independent signalling by a GPCR? Sun et al. observed a dose-dependent switch in the coupling of the β_2-AR from G protein-dependent signalling at low agonist concentrations to G protein-independent signalling at high agonist concentrations (Sun et al., 2007a). Furthermore, Rajagopal et. al. observe that the biochemical effects mediated by the AT_1-i2m receptor mutant parallel the downstream effects of the GRK/β-arrestin-mediated signals at this receptor (Rajagopal et al., 2005). Thus one hypothesis is that G protein-independent signalling

may be initiated by the desensitisation machinery of the cell. However, as many of these GPCR-protein interactions occur independently of β-arrestins, this may not represent a general mechanism for activation of this type of signalling.

1.6.2.4 GPCR dimerisation and signalling

Thus GPCRs are able to interact directly with src and SHP, proteins which were previously considered restricted to the realm of cytokine and RTK signalling. A key element of cytokine and RTK-mediated signalling is receptor dimerisation (Brooks et al., 2007). However, the impact of dimerisation on the signalling of GPCRs is less clear. FRET and BRET (fluorescence and bioluminescence resonance energy transfer respectively) experiments suggest that GPCRs dimerise in the ER following synthesis (Terrillon and Bouvier, 2004). Other evidence, which has also been shown for the GnRH receptor, is that misrouted mutants exert a dominant negative effect on the expression of wildtype receptors at the cell surface, retaining the receptors in the ER and supporting the proposal that dimerisation of receptors occurs early in the trafficking pathway (Brothers et al., 2004; Knollman et al., 2005; Terrillon and Bouvier, 2004). Furthermore, FRET assessment of GPCR dimerisation in the plasma membrane suggests that the GnRH receptors, like other GPCRs, exist as dimers at the cell surface (Cheung and Hearn, 2003; Terrillon and Bouvier, 2004). While early BRET and FRET studies suggested that GPCR dimerisation may be regulated by ligands, it is now currently accepted that GPCRs exist as constitutive dimers at the cell surface (Milligan et al., 2003). While heterodimerisation of GPCRs has been shown to affect the signalling of the constituent receptors, the role of homodimerisation in GPCR signalling is still a matter of debate (Terrillon and Bouvier, 2004). This is particularly pertinent following the observation that purified monomeric β_2-ARs can couple efficiently to G_s in a reconstituted lipid bilayer system (Whorton et al., 2007). Nevertheless, a role for β_2-AR dimerisation has been suggested to facilitate receptor-induced G protein-independent activation of src (Sun et al., 2007a). However, this proposal lacks experimental support. Interestingly, dimerisation is proposed to play a key role in enabling JAK/STAT signalling of chemokine receptors (Mellado et al., 2001; Rodriguez-Frade et al., 2001).

Future work will hopefully provide insight into the broader implications of this phenomenon on GPCR signalling.

1.6.3 Summary

GPCRs have had their signalling repertoire extended to include activation of multiple G protein classes and activation of signalling via G protein-independent mechanisms. This has relevance to LiSS as the structural requirements for activation of these different pathways may be distinct, suggesting that structurally distinct ligands may be able to selectively target desirable signalling pathways. Thus elucidation of the capacity of ligands to activate these pathways and the receptor conformations that facilitate specific signalling is required. Comprehensive understanding of LiSS offers the exciting possibility that GPCR drugs may be developed that have improved specificity at target pathways, while preventing activation of other pathways that may have detrimental side effects.

2.4.8 Homologous binding with the antagonist, Cetorelix, and expression of Tyr$^{6.58}$-mutated GnRH receptors

For technical reasons, ^{125}I-[His^{5}, D-Tyr^{6}]GnRH I was the preferred label in the above assays as ^{125}I-Cetorelix exhibits high non-specific binding. However, the peptide antagonist, Cetorelix exhibited unchanged affinity for the Tyr$^{6.58}$-mutated receptors (Fig.2.3). Thus, the Cetorelix label is useful to assess the affects of Tyr$^{6.58}$ mutation on GnRH receptor expression. Homologous binding assays with Cetorelix were performed (Fig.2.3). The IC_{50} value for Cetorelix at the wildtype receptor was 5.7 ± 1.9 nM, which was similar to that observed at the mutant receptors (Fig.2.3). Mutation of Tyr$^{6.58}$ to Ala did not alter GnRH receptor expression (R_{exp} = 102 ± 5 % of wildtype). However, mutation of the Tyr$^{6.58}$ to Phe and Leu, which have hydrophobic side chains but lack functional groups with hydrogen bonding abilities, decreased receptor expression to 30 ± 5 ($p<0.01$) and 32 ± 3 % ($p<0.01$) of the wildtype level respectively (Fig.2.3). In contrast, mutation of Tyr$^{6.58}$ to Gln increased receptor expression to 228 ± 13 % ($p<0.01$). These data show that receptors with hydrophobic residues in position 6.58, namely the Tyr$^{6.58}$Phe and Tyr$^{6.58}$Leu receptors, have decreased expression. In contrast, receptors which have a side chain that can make hydrogen bonding interactions, specifically the wildtype and Tyr$^{6.58}$Gln receptors, have higher relative expression.

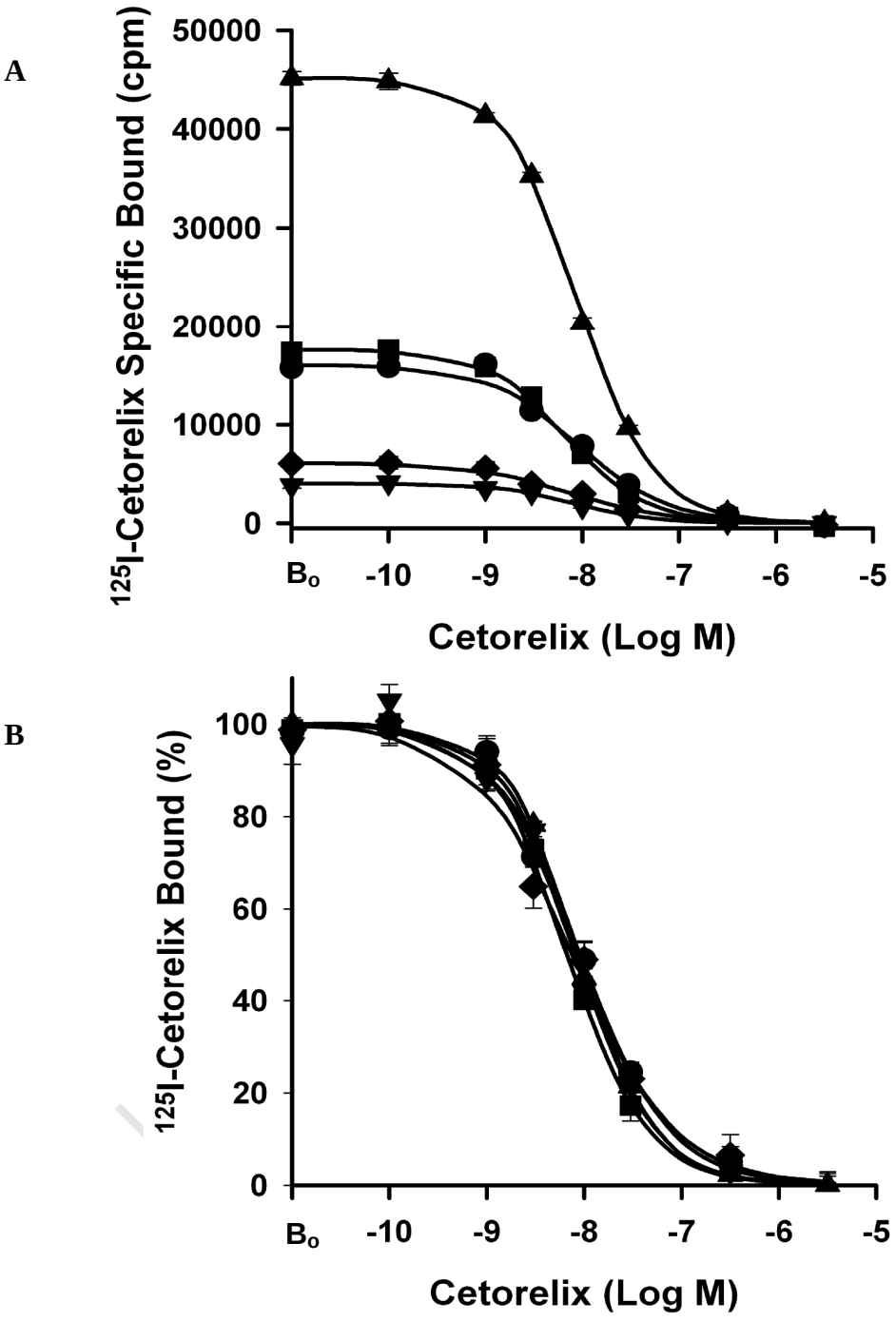


Figure 2.3. Homologous competition binding with Cetorelix at wildtype and Tyr$^{6.58}$-mutated receptors. A, binding curves showing homologous competitive binding of the peptide antagonist Cetorelix at the wildtype and Tyr$^{6.58}$ mutant receptors transiently expressed in COS-7 cells. Results are representative experiments, which were repeated at least three times with similar results. B, normalised binding curves which show no significant differences in receptor affinity for cetorelix at the wildtype and mutant receptors. ●, wildtype; ▼, Tyr$^{6.58}$Phe; ■, Tyr$^{6.58}$Ala; ◆, Tyr$^{6.58}$Leu; ▲, Tyr$^{6.58}$Gln. Points, mean ± standard error of triplicate measurements. B_o, label bound in the absence of competing cold peptide.

2.4.9 Functional responses of Tyr$^{6.58}$-mutated receptors

To evaluate the effects of Tyr$^{6.58}$ mutation on GnRH receptor activation, GnRH-elicited IP responses were determined (Table 2.3). Stimulation of the wildtype GnRH receptor with GnRH I or GnRH II resulted in robust IP responses with E_{max} values approximately 5-fold higher than basal IPs (Fig.2.4). The EC_{50} values for GnRH I and GnRH II at the wildtype receptor were 0.9 ± 0.2 nM and 9.6 ± 2 nM respectively (Table 2.3). Mutation of Tyr$^{6.58}$ to Phe increased EC_{50} values by 57- and 26-fold for GnRH I and GnRH II respectively ($p<0.01$). However, the signalling efficiencies (Q), which consider receptor expression, ligand affinity, potency and E_{max} values, of the Tyr$^{6.58}$Phe receptor for GnRH I and GnRH II were 82 % and 144 % of the wildtype levels respectively (Table 2.3). This suggests that the Tyr$^{6.58}$Phe receptor is as effective, possibly even more effective for GnRH II, in mediating receptor activation than the wildtype receptor. These data indicate the *para*-OH group of Tyr$^{6.58}$ is not important for receptor activation. Mutation of Tyr$^{6.58}$ to Ala produced an even larger decrease in potency with increases in EC_{50} values of 1916- and 202-fold for GnRH I and GnRH II respectively ($p<0.01$). Furthermore, the signalling efficiency of the Tyr$^{6.58}$Ala receptor in response to GnRH I and GnRH II was 23 and 46 % of the wildtype level respectively. This result shows that the Tyr$^{6.58}$Ala receptor is compromised in its ability to mediate receptor activation and suggests that the aromatic ring of Tyr$^{6.58}$ is important for activation of the GnRH receptor by GnRH I and GnRH II. To confirm the validity of these suggestions, the responses of the Tyr$^{6.58}$Phe and Tyr$^{6.58}$Ala receptors were determined in response to [His^{5},D-Tyr^{6}]GnRH I, as it was reasoned that the smaller changes in IC_{50} values for this peptide would yield more accurate results. The signalling efficiencies of the Tyr$^{6.58}$Phe and Tyr$^{6.58}$Ala receptors in response to [His^{5},D-Tyr^{6}]GnRH I were 94 % and 20 % respectively. Thus, these data are consistent with the previous results, showing that the Tyr$^{6.58}$Phe receptor was able to mediate efficient receptor activation, while the Tyr$^{6.58}$Ala receptor was appreciably compromised. This further supports the proposal that the aromatic ring, but not OH group, of Tyr$^{6.58}$ is important in mediating GnRH-elicited receptor activation.

To investigate the proposed molecular requirements of Tyr$^{6.58}$ in receptor activation further, the ability of the Tyr$^{6.58}$Leu and Tyr$^{6.58}$Gln receptors to mediate receptor

activation in response to GnRH I and GnRH II was evaluated. Tyr$^{6.58}$Leu mutation abolished IP signalling in response to GnRH I (Table 2.3; Fig.2.4). This receptor has similar affinity for GnRH I as the Tyr$^{6.58}$Ala receptor (Table 2.1), which has a signalling efficiency of 23% in response to GnRH I. Thus the inability of the Tyr$^{6.58}$Leu receptor to respond to GnRH I is not due to the low affinity of this peptide at the receptor, but is more likely due to the participation of Tyr$^{6.58}$Leu in intramolecular interactions that prevent formation of the active receptor conformations mediating $G_{q/11}$ coupling. In contrast, GnRH II induced an E_{max} of 85 ± 10 % and a signalling efficiency of 116% at the Tyr$^{6.58}$Leu receptor (Table 2.3; Fig.2.4). Thus the Tyr$^{6.58}$Leu receptor has a higher signalling efficiency than the Tyr$^{6.58}$Ala receptor (46%) in response to GnRH II. This shows that Leu in position 6.58 of the GnRH receptor can make compensatory interactions that facilitate efficient receptor activation in response to GnRH II, but not GnRH I. Together, these data show that GnRH I requires an aromatic, rather than hydrophobic, residue in position 6.58, while GnRH II requires a residue with hydrophobic properties for efficient activation.

The Tyr$^{6.58}$Gln receptor had increased E_{max} values relative to the wildtype receptor in response to GnRH I (156 ± 7 %; $p<0.01$) and GnRH II (167 ± 24 %; $p<0.05$), likely due to the increased expression of this receptor. In contrast, the signalling efficiencies of Tyr$^{6.58}$Gln receptor in response to GnRH I and GnRH II were 35 % and 31 % respectively. These are similar to the signalling efficiencies observed at the Tyr$^{6.58}$Ala receptor (Table 2.3). These results indicate that a Gln in position 6.58 is unable to reconstitute the molecular requirements of Tyr$^{6.58}$ necessary for efficient receptor activation. This further supports the data obtained with the Tyr$^{6.58}$Phe receptor which suggests that the hydrogen bonding capabilities of the *para*-OH of Tyr$^{6.58}$ are not necessary to mediate receptor activation. Thus, taken together, these data suggest that Tyr$^{6.58}$ is important in mediating receptor activation in response to both GnRH I and GnRH II. The H-bonding properties of this residue are not important for receptor activation mediated by GnRH I or GnRH II. However, while GnRH I absolutely requires an aromatic ring in position 6.58 for receptor activation, GnRH II tolerates hydrophobic residues in this position.

Table 2.3. GnRH-elicited IP responses of the wildtype and Tyr$^{6.58}$-mutated receptors.
Forty-eight hours following transient transfection of COS-7 cells with wildtype and Tyr$^{6.58}$ mutant receptor constructs and overnight *myo*-[^{3}H]inositol labelling, IP responses to GnRHs were assessed. Signalling efficiencies (Q) of the receptors were determined as described in Experimental Procedures.

	GnRH I [a]				**GnRH II [a]**				**[His^{5}, D-Tyr^{6}]GnRH I [a]**			
Receptor	**Emax (%wt)**	**EC$_{50}$ (nM)**	**EC$_{50}$ Fold change**	**Q (%wt)**	**Emax (%wt)**	**EC$_{50}$ (nM)**	**EC$_{50}$ Fold change**	**Q (%wt)**	**Emax (%wt)**	**EC$_{50}$ (nM)**	**EC$_{50}$ Fold change**	**Q (%wt)**
Wildtype	100	0.9 ± 0.2	1.0	**100**	100	9.6 ± 2	1.0	**100**	100	0.7 ± 0.1	1.0	**100**
Tyr$^{6.58}$Phe	94 ± 7	51 ± 10**	57	**82**	87 ± 12	254 ± 27**	26	**144**	107 ± 3	4.7 ± 0.4**	6.7	**94**
Tyr$^{6.58}$Ala	72 ± 6**	1724 ± 146**	1916	**23**	82 ± 18	1940 ± 175**	202	**46**	88 ± 6	73 ± 10**	104	**20**
Tyr$^{6.58}$Leu	<10	ud	ud	**ud**	85 ± 10	1674 ± 208**	174	**116**				
Tyr$^{6.58}$Gln	156 ± 7**	227 ± 26**	252	**35**	167 ± 24*	1607 ± 162**	167	**31**				

[a] Data are the mean ± S.E. of at least three independent experiments performed in triplicate.
ud, undetectable IP response.
*****, p<0.05; ******, p<0.01; Students *t* test; significantly different from the wildtype receptor.

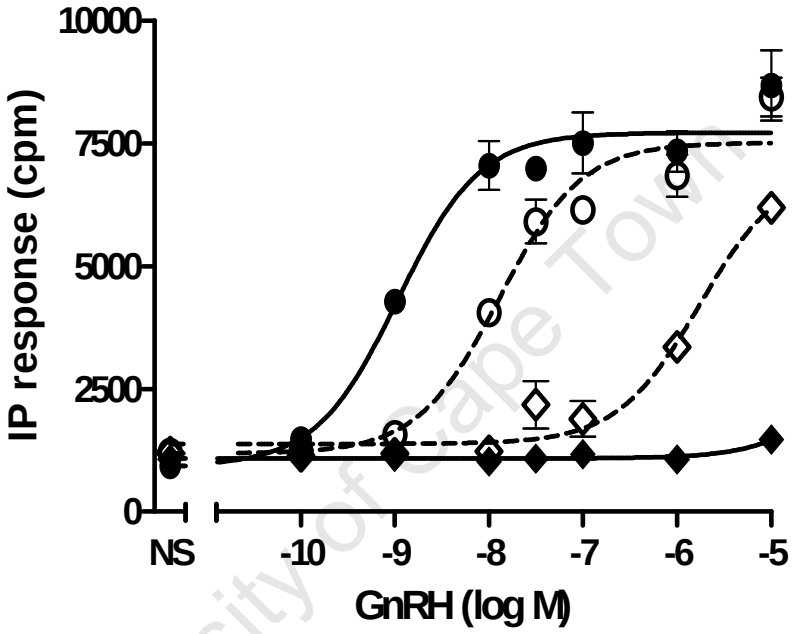


Figure 2.4. GnRH-elicited IP responses at the wildtype and Tyr$^{6.58}$Leu mutant receptors. Forty-eight hours following transient transfection of COS-7 cells with wildtype (●/○) and Tyr$^{6.58}$Leu (♦/◇) receptors and overnight [^{3}H]-*myo*-inositol labelling, IP responses to GnRH I (solid symbols ●/♦) and GnRH II (open symbols ○/◇) were determined and sigmoidal dose-response curves fitted (solid and dashed lines respectively). This figure is representative of three independent experiments performed in triplicate. NS, non-stimulated/basal IP responses.

2.5 Discussion

An exciting emerging field of GPCR research is directed towards the understanding of the molecular requirements underlying the ability of structurally distinct ligands to induce different receptor active conformations that have distinct downstream signalling capabilities (Perez and Karnik, 2005; Urban et al., 2007; Weinstein, 2005). This enquiry of research has therapeutic relevance in the rational design of GPCR drugs with increased specificity for desired signalling pathway and reduced side-effects. The two endogenous ligands, GnRH I and GnRH II, have been identified as having differential signalling abilities at the human GnRH receptor (Lu et al., 2005; Millar et al., 2008). The GnRHs differ at positions 5, 7 and 8 in the decapeptide sequence. Here, a comprehensive study was performed to investigate the differential ligand-receptor molecular interactions between Tyr^{5} of GnRH I and His^{5} of GnRH II, and Tyr$^{6.58}$ of the GnRH receptor. Furthermore, the importance of Tyr$^{6.58}$ in receptor expression and activation was determined.

2.5.1 Tyr^{5} of GnRH I is proposed to interact with Tyr$^{6.58}$ of the GnRH receptor

Sequential substitution of Tyr^{5} with Phe- and Ala-substituted GnRH I analogues produced 2- and 127-fold decreases in affinity of the peptide for the wildtype receptor respectively, indicating the importance of the aromatic ring, but not the *para*-OH group, of Tyr^{5} in GnRH I binding to the GnRH receptor (Table 2.1). Mutation of Tyr$^{6.58}$ to Phe and Ala induced 4.9- and 332-fold decreases in affinity for GnRH I revealing the importance of both the OH and aromatic ring of Tyr$^{6.58}$ in GnRH I binding (Table 2.1). Evaluation of the affinities of position 5-substituted GnRH I analogues for Tyr$^{6.58}$-mutated receptors have led to the proposal that the OH and aromatic ring of Tyr$^{6.58}$ interact with the aromatic ring of Tyr^{5} of GnRH I. This is suggested from the experimental data indicating that Tyr$^{6.58}$Phe and Tyr$^{6.58}$Ala receptors exhibited smaller affinity decreases for Ala^{5}-substituted GnRH I analogues relative to native GnRH I (25- and 3.2-fold respectively) compared with the wildtype receptor (127-fold). The interaction of Tyr$^{6.58}$ and Tyr^{5} of GnRH I suggested by these experimental data support the predictions of independently constructed molecular models of the GnRH receptor, based on the structure of rhodopsin, which incorporated previously delineated GnRH I binding interactions (Hovelmann et al., 2002; Millar et al., 2004).

In our molecular model of the GnRH receptor, Tyr$^{6.58}$ and Tyr^{5} are arranged in a T-shaped aromatic stacking interaction, where the OH and aromatic ring of Tyr$^{6.58}$ point towards the aromatic ring of Tyr^{5} of GnRH I (Fig.2.2). This specialised aromatic interaction is proposed to consist of van der Waals, hydrophobic and electrostatic forces, where the relative contribution may depend on the geometry of the aromatic rings relative to each other (Waters, 2002; Waters, 2004). The aromatic nature of the interaction between Tyr$^{6.58}$ and Tyr^{5} proposed by the molecular model is supported by the experimental data where the Tyr$^{6.58}$Leu receptor, which has a hydrophobic, but not aromatic, side chain in position 6.58, had similar affinity for GnRH I as the Tyr$^{6.58}$Ala receptor and considerably lower affinity than the Tyr$^{6.58}$Phe receptor, supporting the importance of an aromatic ring at this position in the receptor in GnRH I binding.

2.5.2 His^{5} of GnRH II is proposed to interact with Tyr$^{6.58}$ of the GnRH receptor

While the above experimental data supports an interaction between Tyr^{5} of GnRH I and Tyr$^{6.58}$ of the GnRH receptor, it was considered likely that the interactions of His^{5} of GnRH II would differ from those of GnRH I. Nevertheless, similar to the data obtained with GnRH I, mutation of Tyr$^{6.58}$ to Phe and Ala induced an 8- and 82-fold decrease in affinity for GnRH II respectively (Table 2.2), indicating that the OH group and aromatic ring of Tyr$^{6.58}$ are also important for GnRH II binding. Similarly, substitution of His^{5} of GnRH II with Ala produced a large decrease (177-fold) in affinity for the wildtype receptor showing that position 5 of GnRH II, like GnRH I, is important for high affinity binding interactions. The interaction of the His^{5} side chain with the OH group and aromatic ring of Tyr$^{6.58}$ is suggested by the experimental results indicating that the Tyr$^{6.58}$Phe and Tyr$^{6.58}$Ala receptors exhibited smaller changes in affinity for [Ala^{5}]GnRH I (14- and 4.5-fold respectively) than the wildtype receptor (177-fold). Thus these results suggest that His^{5} of GnRH II, like Tyr^{5} of GnRH I, also interacts with Tyr$^{6.58}$ of the GnRH receptor. This supports the proposal that receptor binding contacts for GnRH II overlap with those of GnRH I (Millar et al., 2004).

In our molecular model of GnRH II docked to the GnRH receptor, His^{5} of GnRH II, like Tyr^{5} of GnRH I, also forms an aromatic interaction with Tyr$^{6.58}$ of the GnRH receptor. The involvement of His^{5} of GnRH II in an aromatic interaction is supported by the observation that substitution of His^{5} of GnRH II with Tyr, which also exhibits aromatic properties, retained high affinity peptide binding (Table 2.2). Further support is that the Tyr$^{6.58}$Leu receptor did not have comparable affinity for GnRH II as the Tyr$^{6.58}$Phe receptor, emphasising the importance of an aromatic ring in this position for high affinity GnRH II binding (Table 2.2). A notable difference between GnRH I and GnRH II binding, proposed by the molecular model, is that His^{5} of GnRH II and Tyr$^{6.58}$ form a parallel offset stacking interaction that differs from the T-shaped stacking interaction observed in the GnRH I interaction between Tyr^{5} and Tyr$^{6.58}$. This orientation of residues in an aromatic interaction has increased components of van der Waals and hydrophobic interactions (Waters, 2002). Interestingly, this arrangement is supported by the experimental data where the hydrophobic characteristics of the Tyr$^{6.58}$Leu mutation enabled some reconstitution of GnRH II binding affinity compared with the Tyr$^{6.58}$Ala receptor. In contrast, this partial rescue of peptide affinity by the Tyr$^{6.58}$Leu receptor was not observed with GnRH I. This shows that the molecular requirements of GnRH I and GnRH II interaction with Tyr$^{6.58}$, while similar, are distinct as there is a greater hydrophobic component to the interaction of position 5 of GnRH II with Tyr$^{6.58}$ of the receptor, compared with GnRH I. This thus supports the predictions of the molecular model where Tyr^{5} and His^{5} interact with different rotamer conformations of Tyr$^{6.58}$ by aromatic interactions with distinct geometries. The differential interactions of Tyr^{5} of GnRH I and His^{5} of GnRH II with Tyr$^{6.58}$ are also supported by the observation that Tyr$^{6.58}$Ala mutation induced larger decreases in affinity for GnRH I (332-fold) than for GnRH II (82-fold).

2.5.3 Tyr$^{6.58}$ mutation induces large decreases in affinity for GnRH I and GnRH II, but not [His^{5}, D-Tyr^{6}]GnRH I

Aromatic interactions are biologically relevant forces that contribute to stabilisation of protein structure and direct the specificity of intramolecular interactions thereby facilitating protein folding into the required protein conformation (Waters, 2002; Waters, 2004). However, an aromatic interaction is a weak force and the loss of a

single isolated interaction pair would not be expected to significantly effect the interaction between two proteins/peptides (Waters, 2002; Waters, 2004). In contrast, my experimental data show that removal of the aromatic ring at Tyr$^{6.58}$ led to large changes in affinity for GnRHs. Specifically, when Tyr$^{6.58}$ was mutated to Ala this led to a 332-fold and 82-fold reduction in affinity for GnRH I and GnRH II respectively. One explanation for these large effects on ligand affinity is that Tyr$^{6.58}$ makes interactions with other residues in the ligand or in the receptor, in addition to its partnered interaction with position 5 of the GnRH. However, the experimental results show that the Tyr$^{6.58}$ mutation exhibited only relatively small changes in affinity for Ala^{5}-substituted GnRH I and GnRH II analogues (8.3-fold and 2.1-fold respectively). This suggests that the large decrease in affinity for GnRH I and GnRH II (of 332- and 82-fold respectively) induced by Tyr$^{6.58}$ mutation to Ala is primarily due to the loss of its interaction with position 5 of the GnRH.

A second explanation for the large decrease resulting from the loss of the interaction between Tyr$^{6.58}$ and position 5 of the GnRH is that this interaction contributes to the formation of subsequent GnRH-receptor contacts. Recent studies suggest that ligand-GPCR interactions occur in a sequential manner and that initial interactions synergistically contribute to subsequent ligand-receptor contacts (Del Carmine et al., 2004; Kobilka, 2007). Experimental data which provides mechanistic insight into how this may occur in the GnRH receptor is the observation that [His^{5}, D-Tyr^{6}]GnRH I exhibited smaller changes in affinity for Tyr$^{6.58}$-mutated receptors than the other GnRH analogues (Table 2.1; Table 2.2). The Tyr$^{6.58}$Phe had similar affinity for [His^{5}, D-Tyr^{6}]GnRH I as the wildtype receptor, while the Tyr$^{6.58}$Ala, Tyr$^{6.58}$Leu and Tyr$^{6.58}$Gln receptors all exhibited small ~10-fold decreases in affinity. Thus [His^{5}, D-Tyr^{6}]GnRH I is less reliant on the interaction of position 5 of the ligand with Tyr$^{6.58}$ than other GnRH analogues. An important distinguishing feature of [His^{5}, D-Tyr^{6}]GnRH I compared with other GnRHs is that this peptide is already constrained in the high affinity peptide conformation required for GnRH binding to the receptor (Barran et al., 2005). GnRHs are proposed to bind to the receptor in a βII'-type turn conformation (Barran et al., 2005). However, in solution GnRH peptides are able to assume a multitude of conformations. Thus initial ligand-receptor contacts between position 5 of the ligand and Tyr$^{6.58}$ may facilitate

configuration of the ligand into the high affinity conformation that facilitates subsequent ligand-receptor contacts. [His^5, D-Tyr^6]GnRH I, which is intrinsically constrained in the high affinity conformation, would thus be less dependent on this interaction for high affinity binding. This proposal is further supported by previous experimental data which showed that mutation of Asp$^{7.32}$ of the GnRH receptor, which is proposed to interact with Arg^8 of GnRH I, also induced considerably smaller decreases in affinity for a range of constrained GnRH analogues compared with other GnRH peptides (Fromme et al., 2001). This phenomenon is specific to these residues in the mid-region of the GnRH peptide as mutation of Asp$^{2.61}$ and Asn$^{2.65}$, which interact with His^2 and Gly-NH_2 in the N- and C-termini of the peptide respectively, exhibit similar decreases in affinity for native and constrained GnRH analogues (Davidson et al., 1996; Flanagan et al., 2000). Thus these results support a binding scheme where one end/terminus of the GnRH peptide binds to the receptor, followed by receptor interactions with residues in the mid-region of the ligand that facilitate configuration of the bioactive conformation of the ligand, thereby enabling subsequent interactions of the opposite terminus of the peptide with the receptor.

2.5.4 The peptide antagonist, Cetorelix, exhibited unchanged affinity for the Tyr$^{6.58}$ mutant receptors

The peptide antagonist, Cetorelix, also has a Tyr in position 5. However, it had unchanged affinity for the Tyr$^{6.58}$ mutant receptors compared with the wildtype receptor (Fig.2.3). This result shows that Tyr^5 of Cetorelix does not interact with Tyr$^{6.58}$. Thus position 5 of Cetorelix binds differentially to the GnRH receptor compared with the agonists, GnRH I and GnRH II. This result is not surprising as Cetorelix is only 50% homologous with GnRH I and is thus likely to bind to the receptor in a different conformation (Millar et al., 2004). The ability of the agonists, GnRH I and GnRH II, to bind to Tyr$^{6.58}$, but not the antagonist, Cetorelix, is consistent with the proposed role of this residue in receptor activation, as measured by IP assays, discussed below.

2.5.5 Tyr$^{6.58}$ of the GnRH receptor is important for receptor expression

In addition to its importance in GnRH I and GnRH II binding, mutation of Tyr$^{6.58}$ altered receptor expression. While mutation of Tyr$^{6.58}$ to Ala did not affect receptor

expression, mutation to residues with hydrophobic side chains, Phe and Leu, decreased receptor expression to ~30% of the wildtype level (Fig.2.3). These data suggest that hydrophobic residues in position 6.58 destabilise receptor structure thereby preventing efficient receptor trafficking to the cell surface. The higher relative expression of the wildtype receptor suggests that this effect is corrected by the presence of a functional group that can make H-bonds at this position. Thus, the OH of Tyr$^{6.58}$ is likely to make intramolecular interactions that stabilise receptor conformation. This is further supported by the observation that the Gln receptor, which does not have a hydrophobic destabilising influence, but retains the H-bonding abilities at position 6.58, had 228 ± 13 % increased expression relative to the wildtype receptor.

2.5.6 Tyr$^{6.58}$ of the GnRH receptor is important for receptor activation

The ability of Tyr$^{6.58}$ intramolecular interactions to alter receptor conformation, suggested by the above data exploring receptor expression, is further supported by the experimental data which shows that Tyr$^{6.58}$ is important for receptor activation, which requires a conformational change in receptor structure. The effects of Tyr$^{6.58}$ on receptor activation were determined using IP assays, which provide a measure of $G_{q/11}$ activation by the GnRH receptor. Mutation of Tyr$^{6.58}$ to Phe produced signalling efficiencies of 82 % and 144 % of the wildtype levels for GnRH I and GnRH II respectively (Table 2.3). This suggests that the OH group of Tyr$^{6.58}$ is not important for receptor activation in response to GnRH I and GnRH II. However, mutation of Tyr$^{6.58}$ to Ala induced a large change in signalling efficiencies to 23 % and 46 % of wildtype levels in response to both GnRH I and GnRH II (Table 2.3). Thus, the aromatic ring of Tyr$^{6.58}$ is important in facilitating receptor activation by both GnRH I and GnRH II. Interestingly, while the Tyr$^{6.58}$Leu receptor has similar affinity as the Tyr$^{6.58}$Ala receptor for GnRH I, Tyr$^{6.58}$Leu mutation abolished IP signalling in response to GnRH I (Fig.2.4). This result suggests that Tyr$^{6.58}$Leu makes intramolecular interactions in the GnRH I-stabilised receptor conformation that prevent formation of an active $G_{q/11}$-coupled conformation. In contrast, GnRH II-elicited IP signalling was improved at the Tyr$^{6.58}$Leu receptor (116 %) compared with the Tyr$^{6.58}$Ala receptor (46%). Together, these results suggest that Tyr$^{6.58}$Leu participates in different intramolecular interactions that stabilise different receptor

active conformations in response to GnRH I and GnRH II. This supports the above proposal that GnRH I and GnRH II bind to different rotamer conformations of Tyr$^{6.58}$ of the receptor. How different rotamer conformations of Tyr$^{6.58}$ differentially affect receptor conformation and downstream signalling requires further investigation. However, one suggestion is that the rotamer configuration of Tyr$^{6.58}$ affects the conformations of other residues in TM6 by a "domino effect", which may differentially affect the rotamer toggle switch and thus the active conformation of the receptor (Schwartz et al., 2006; Shi et al., 2002).

In summary, the experimental results suggest that the OH and aromatic ring of Tyr$^{6.58}$ of the human GnRH receptor interact with the aromatic rings of Tyr^{5} of GnRH I and His^{5} of GnRH II. Our molecular models, supported by the experimental results, indicate that position 5 of GnRH I and GnRH II engages with distinct rotamer conformations of Tyr$^{6.58}$, by different aromatic stacking interactions. The experimental data show that Tyr$^{6.58}$ mutation altered receptor expression and thus must be spatially positioned to make intramolecular interactions that can alter receptor conformation. Furthermore, Tyr$^{6.58}$ is important in facilitating receptor activation. The differential responses of the Tyr$^{6.58}$Leu receptor to GnRH stimulation suggest that the interactions of Tyr$^{6.58}$, necessary for receptor activation, differ for GnRH I and GnRH II. These data thus support the proposal that GnRH I and GnRH II stabilise different receptor active conformations that may contribute to the distinct pharmacological and signalling profiles of these two ligands at the GnRH receptor.

3 Chapter 3: Investigation of the ability of the GnRH receptor to couple to the G_i family of G proteins

3.1 Abstract

In reproductive cancer cells, the GnRH receptor mediates anti-proliferative signalling that does not correlate with activation of classical $G_{q/11}$ signalling. Several reports have suggested the involvement G_i in these effects. This has led to the proposal that the GnRH receptor mediates anti-proliferative signalling by direct coupling to G_i. However, experimental verification is required. In the present study, direct activation of G_i by the GnRH receptor was investigated using the [^{35}S]GTPγS binding assay. Using the [^{35}S]GTPγS assay with rapid filtration, GnRH receptor-mediated activation of G_i was not detected, despite robust agonist-stimulated G_i coupling observed at the CCR5 receptor. Next, using the more sensitive [^{35}S]GTPγS binding assay, the Scintillation Proximity Assay (SPA), GnRH receptor-G_i coupling was also not observed. Detection of agonist-stimulated G_i activation by the M_2 muscarinic receptor and $G_{q/11}$ activation by the GnRH receptor provided verification of the functionality of the SPA. Finally, a reconstituted baculovirus-infected system enabling high expression of the GnRH receptor and G_i in a cell background of low endogeneous G proteins also failed to facilitate detection of GnRH receptor-mediated G_i activation. In this system, detection of G_i activation by CCR5 verified that the assay was operational. These results show that the GnRH receptor is not able to directly activate G_i and thus negates the proposal that the proximal receptor-mediated events of GnRH receptor anti-proliferative signalling are mediated by GnRH receptor-G_i coupling.

3.2 Introduction

The GnRH receptor mediates anti-proliferative and proapoptopic signalling in reproductive cancer cells (Grundker and Emons, 2003; Grundker et al., 2001; Kraus et al., 2006; Maiti et al., 2005; Maudsley et al., 2004). However, activation of this pathway does not correlate with activation of classical $G_{q/11}$ signalling by the GnRH receptor. Specifically, GnRH I and GnRH II exhibit a reversal of potency at the two pathways and classical GnRH receptor antagonists, which do not activate $G_{q/11}$, are able to induce anti-proliferative signalling at the GnRH receptor (Grundker and Emons, 2003; Millar et al.,

2008). These data suggest that another GnRH receptor-mediated pathway is responsible for these anti-proliferative effects. Several researchers have reported the importance of G_i signalling in GnRH receptor-mediated anti-proliferative signalling and thus it has been proposed that the anti-proliferative effects of the GnRH receptor are mediated by its ability to couple to this alternative G_i, rather than $G_{q/11}$, protein family (Grundker and Emons, 2003; Grundker et al., 2001; Limonta et al., 1999; Millar et al., 2004; Millar et al., 2008).

However, there is disparity concerning whether the GnRH receptor is able to couple directly to G_i proteins. Most studies have used analysis of second messenger generation and pertussis toxin to infer the involvement of G_i in GnRH receptor signalling (Kimura et al., 1999; Limonta et al., 1999; Maudsley et al., 2004; Sim et al., 1995). As these methods of analysis investigate signalling distal to receptor-G protein coupling and are subject to signal cross-talk (Birnbaumer, 2007), they do not provide conclusive evidence for a direct GnRH receptor-G_i interaction. Other methods used to infer GnRH receptor activation of G_i have included receptor-G protein cross-linking studies (Grundker et al., 2001), agonist-stimulated palmitoylation and G protein release (Krsmanovic et al., 2003; Stanislaus et al., 1998) and agonist-stimulated protection of G_i from PTX-catalysed ADP ribosylation (Limonta et al., 1999). In contrast, a study of GnRH receptor-induced photolabelling of G_q and G_i with [α-^{32}P]GTP azidoanilide detected only agonist-induced labelling of G_q and not G_i, suggesting that the GnRH receptor does not directly couple to G_i (Grosse et al., 2000).

In the present study, to address this dispute regarding GnRH receptor-G_i coupling, the [^{35}S]GTPγS binding assay was performed with GnRH receptor-expressing membranes. This assay measures the most proximal step of receptor-G protein coupling, specifically the receptor-stimulated exchange of GDP for GTP on the Gα protein (Harrison and Traynor, 2003; Lazareno, 1999; Milligan, 2003; Weiland and Jakobs, 1994). Membranes, containing the GPCR and G proteins, are incubated with the radioactively-labelled non-hydrolysable GTP analogue, [^{35}S]GTPγS, in the presence or absence of

agonist. Agonist stimulation leads to receptor-induced GDP/GTP exchange and the activated G proteins are thus labelled with [^{35}S]GTPγS. The non-hydrolysable nature of the analogue prevents the loss of signal by the intrinsic G protein GTPase activity. This well-established method for measurement of GPCR-G protein coupling has a number of advantages. Firstly, in addition to providing a direct measure of G protein activation, the assay is performed in membranes and thus the signal cannot result from downstream signalling cross-talk. Furthermore, the assay provides a quantitative measure of receptor-induced G protein activation.

Several variations of the [^{35}S]GTPγS binding assay were explored. These methods confer different advantages and vary in the mechanism whereby, following the initial incubation step, the G protein-bound [^{35}S]GTPγS is separated from the unbound molecules (Fig.3.1). The simplest method involves capture of the membranes (containing G protein-bound [^{35}S]GTPγS) by rapid filtration through glass fibre filters. This is the least sensitive of the assays, but is cost-effective and technically undemanding. A second method involves capture of the G proteins by immunoprecipitation. This assay has the advantage of enabling the use of G protein subtype-specific antibodies, thus providing a more specific, in addition to more sensitive, signal. The Scintillation Proximity Assay (SPA) involves a further modification of the immunoprecipitation method (DeLapp et al., 1999). Here, a scintillation bead is targeted to the antibody bound-G protein. If the G protein is bound to [^{35}S]GTPγS, the proximity of the radioactive molecule to the scintillation bead facilitates the generation of light. This assay therefore precludes the necessity to separate bound and unbound [^{35}S]GTPγS and is thus particularly amenable to high throughput assays.

Optimisation of the [^{35}S]GTPγS binding assay requires variation of a number of factors relevant to the assay incubation conditions and optimal conditions will differ depending on the GPCR, G protein subtype and cell line investigated (Harrison and Traynor, 2003; Lazareno, 1999). The optimal time and temperature of the incubation step require

investigation. Additionally, the effects of altering Mg^{2+}, Na^{+} and GDP concentrations need to be assessed. Mg^{2+} ions promote [^{35}S]GTPγS binding by enabling the GDP/GTP exchange on the G protein (Birnbaumer, 2007; Harrison and Traynor, 2003). In contrast, both Na^{+} ions and GDP improve the signal-to-noise ratio by decreasing basal [^{35}S]GTPγS binding (Harrison and Traynor, 2003). Na^{+} is proposed to facilitate this effect by reducing GPCR constitutive activity, while GDP binds with higher affinity to the inactive, versus active, G protein (Harrison and Traynor, 2003). Furthermore, the most effective antibody concentration needs to be assessed when performing immunoprecipitation/SPA experiments. In this study, an exhaustive investigation of differing assay conditions was performed in order to investigate the ability of the GnRH receptor to couple to G_i using the [^{35}S]GTPγS binding assay.

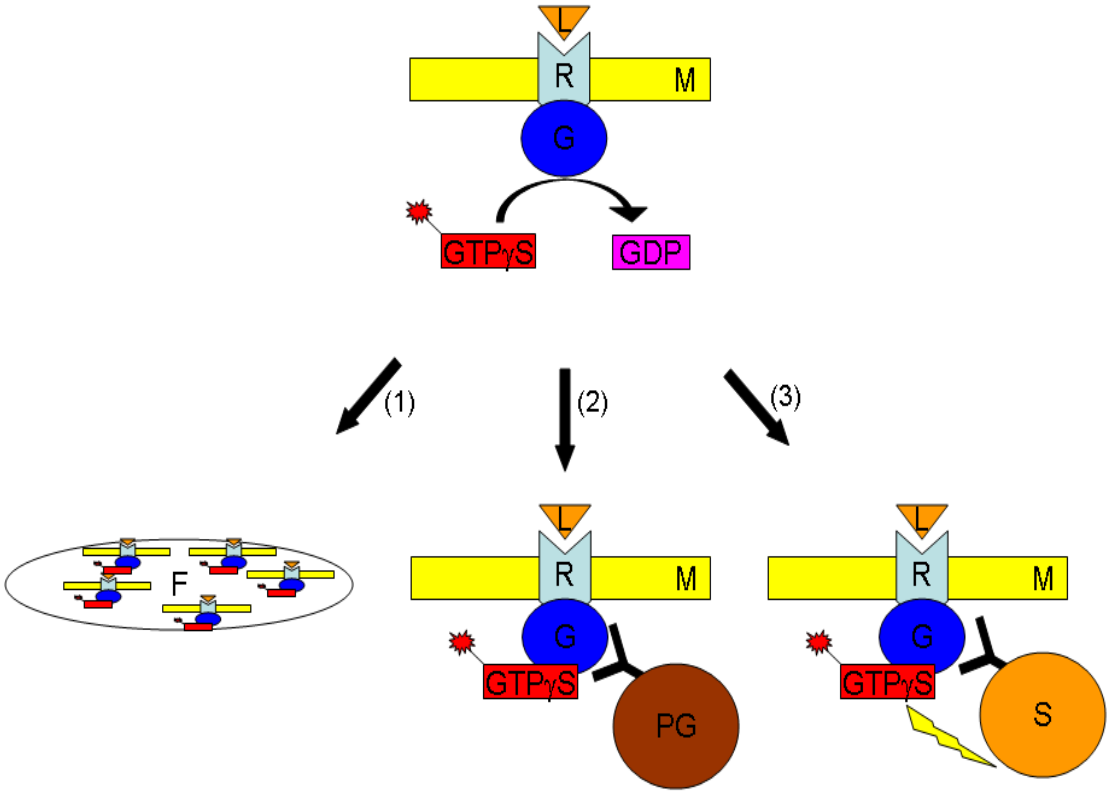


Figure 3.1. Schematic representation of the principles and variations of the [^{35}S]GTPγS binding assay. Membranes (M) containing the GPCR (R) and G proteins (G) of interest are incubated with radioactively-labelled [^{35}S]GTPγS in the presence or absence of the GPCR ligand (L). Ligand-induced activation of the GPCR facilitates GDP/GTP exchange on the G protein and thus [^{35}S]GTPγS becomes bound to the activated G protein, providing a measure of GPCR-mediated G protein activation. Separation of G protein-bound, from unbound, [^{35}S]GTPγS can be performed in several ways (1-3). Firstly, it can occur by rapid filtration through GF/C glass fibre filters (F) (1). To obtain a more sensitive and specific signal, G proteins can be isolated using G protein-specific antibodies (2-3). The antibody-bound G proteins can then be separated with Protein G-sepharose (PG) (2). Alternatively, use of the SPA bead (S) precludes the necessity to separate bound and unbound [^{35}S]GTPγS, because the bead only emits a signal when attached to a [^{35}S]GTPγS-bound G protein. See text for details.

3.3 Experimental Procedures

3.3.1 Materials

Gi_{1-3} constructs in pcDNA3 and antibodies against the C-termini of $G_{i1/2}$ and G_{i3} were generated and generously provided by Dr. Graeme Milligan. GnRH receptor constructs in pcDNA3.1 were constructed in our laboratory. The CCR5 construct was generously provided by Dr. Vanessa Hayes. The $G_{q/11}$(C-19) antibody was from Santa Cruz Biotechnology. Anti-IgG-coated SPA beads and Protein G sepharose were obtained from Amersham, pansorbin cells from Calbiochem and Complete EDTA-free Protease inhibitor cocktail tablets from Roche Applied Sciences. G418/Geneticin and carbachol were purchased from Sigma. Sf21 cells were from ATCC. Components (vectors (see below) and DH10Bac cells) for generation of baculovirus constructs were from Invitrogen. [^{35}S]GTPγS (1000-1250 Ci/mmol) was purchased from Amersham.

3.3.2 Cloning

In order to generate baculoviruses, the following constructs were subcloned into the appropriate vectors using PCR. The CCR5 and human and rat GnRH receptors were subcloned into the pFastbac vector. Additionally, the different Gα protein subunits (Gi_1, Gi_2 and Gi_3) were also subcloned into pFastbac1. The $G\beta_1$ and $G\gamma_2$ subunits were cloned into the pFastbacDual vector so that all cells infected with the virus would always express both subunits. The constructs were verified by dideoxy sequencing.

3.3.3 Cell culture and generation of stable cell lines

Cell culture of mammalian cells was performed as described in chapter 2. SCL60 cells, HEK293 cells stably expressing the rat GnRH receptor, were generated in our laboratory previously (Maudsley et al., 2004). CHO-M_2 muscarinic receptor stable cell lines were obtained by Zhi-liang Lu from his previous laboratory. HEK293 cells were transfected with the CCR5 receptor and the human GnRH receptor using Fugene6 (Roche) and stably transfected cells were selected for using 800μg/ml G418.

Sf21 insect cells were grown in suspension culture at 27^{o}C in Sf900II medium with L-glutamine (Gibco) containing 10%FBS (Delta Bioproducts) in the presence of Penicillin and Streptomycin (2 mg/ml streptomycin sulphate; 4000 U/ml Benzylpenicillin).

3.3.4 Generation of baculovirus

Baculoviruses were generated according to manufacturer's instructions (Gibco). Viruses were amplified and viral titres were determined by end-point dilution as described previously (O'Reilly et al., 1994).

3.3.5 Preparation of membranes for binding and GTPγS assays

Either stably-transfected cells or baculovirus-infected cells were used for membrane preparations. For Sf21 cells, baculoviruses were allowed to infect cells at a multiplicity of infection (MOI) of 3:6:6 for receptor:Gα:G$\beta_1\gamma_2$. However, various MOI ratios were investigated to determine this optimal concentration. Cells were allowed to express protein for 48h and membranes were harvested in the following way. Cells were collected in a harvesting buffer (20mM HEPES; 100mM EDTA; pH 7.5) and ruptured with 20 strokes of a glass dounce homogeniser. Nuclei and unbroken cells were separated with a low speed centrifugation step of 200*g* for 15 minutes. The resultant supernatant was then subjected to a high speed spin at ~40 000*g* for 45 minutes and resuspended in the appropriate buffer depending on assay. To ensure optimal assay conditions, membranes were freshly prepared rather than stored at -80^{o}C as it is our experience that the GnRH receptor is not stable under these conditions (Colleen Flanagan, unpublished observations). Membrane concentrations were determined by the method described by Lowry et al. (Lowry et al., 1951).

3.3.6 Iodination of MIP-1β

A 7.8µg/10µl aliquot of macrophage inflammatory protein 1-beta (MIP-1β) (Cytolab Ltd) was mixed with 10µl of 1mCi $Na^{125}I$ (Amersham) and 15µl of 0.5M phosphate buffer (pH 7.4). Chloramine T (10µl of 1mg/mL in 0.5M phosphate buffer) was added and the reaction allowed to proceed for 1 minute. The reaction was stopped by adding 50µl of sodium metabisulfide (1mg/mL in phosphate buffer). The reaction mixture was applied onto a G-25 column and eluted with PBS containing 0.1% BSA. Fractions were collected after every 90sec. The first peak of eluted fractions was aliquotted and stored. The GnRH label was produced as described in chapter 2.

3.3.7 Radioligand binding assays for CCR5 and the GnRH receptor

Membranes expressing the appropriate receptor were incubated in a binding buffer (GnRH receptor binding buffer (10mM HEPES; 1mM EDTA; pH 7.4; 0.1%BSA); CCR5 binding buffer (50mM HEPES; pH 7.4; 1mM $CaCl_2$; 5mM $MgCl_2$)), the appropriate radiolabel of 50 000cpm per tube (^{125}I-MIP-1β or ^{125}I[His^5,D-Tyr^6]GnRH I) and various concentrations of competing cold ligand. Tubes were incubated at 4^{o}C for 16h (for the GnRH receptor) or 27^{o}C for 1h (CCR5). Incubated tubes were filtered through GF/C filters (Whatman) using a Brandel Harvester. The GF/C filters were pre-soaked in 1%BSA (for CCR5) or 1%PEI (for the GnRH receptor). Filters were washed twice with ice-cold washing buffer (0.01%PEI for GnRH; 50mM HEPES, 1mM $CaCl_2$, 5mM $MgCl_2$ and 0.5M NaCl for CCR5). Thereafter filters were counted with a gamma counter.

3.3.8 [^{35}S]GTPγS binding assays

Membranes expressing the receptor of interest (~75µg of protein/well for SPA and similar concentrations for other experiments) were incubated in the presence or absence of ligand and 0.2nM/100nCi [^{35}S]GTPγS in a GTPγS assay buffer (5mM $MgCl_2$; 100mM NaCl; 20mM HEPES; 1mM EDTA; 1mM dithiothreitol) for various times at 30^{o}C (25^{o}C for SPA). All of the above-mentioned components were varied to investigate

their effects on the reaction. Figure legends provide information of the concentrations of these factors specific to that experiment. The reaction was stopped by addition of a stopping buffer (which was Buffer A (ice-cold water) for rapid filtration experiments and Buffer B (5mM $MgCl_2$; 100mM NaCl; 20mM HEPES; pH 7.4) for immunoprecipitation experiments). For rapid filtration experiments, the reaction mix was filtered through GF/C filters, which were washed twice with ice-cold water as described previously (Lazareno, 1999). The filters were counted using liquid scintillation counting. For immunoprecipitation experiments, the reaction mix was centrifuged (14 000rpm for 15 minutes) and membranes were solubilised in a solubilisation buffer (100mM Tris-HCl; 200mM NaCl; 1mM EDTA; 1.25% NP-40; pH 7.4 and a Protease inhibitor cocktail tablet) at 4^{o}C for 1 hour. Samples were precleared with pansorbin cells for 1 hour and immunoprecipitated with an appropriate antibody conjugated to protein G sepharose overnight. The immunoprecipitated complexes were washed twice with solubilisation buffer and counted using liquid scintillation spectrometry. For SPA, the assay (performed in 96-well plates) proceeded as described previously (DeLapp et al., 1999). Briefly, membranes were solubilised with a 0.3% NP-40 solution for 30 minutes. Thereafter, antibodies (used at concentrations ranging from 1/440 for $Gi_{1/2}$ antibody to 1/1100 for $G_{q/11}$ antibody- optimal concentration needs to be determined for each antibody) and SPA beads were added and incubated for a further 3 hours. Plates were centrifuged at 3000rpm for 10 minutes and counted in a beta-counter. Data were plotted and analysed using GraphPad Prism 4 (Graphpad software Inc.).

3.4 Results

3.4.1 Investigation of GnRH receptor-G_i coupling using the [^{35}S]GTPγS binding assay with rapid filtration

HEK293 cells stably expressing the rat GnRH receptor (SCL60 cells) were selected for use in the [^{35}S]GTPγS binding assay. The reasons were two-fold. Firstly, this cell line expresses high levels of the GnRH receptor, as determined by radioligand binding assays (data not shown). This criterion is important in order to ensure a robust signal, as G protein activation is not subject to the amplification observed at more distal signalling molecules. Secondly, previous studies in this cell line reported potent and efficacious induction of anti-proliferative signalling by the GnRH receptor, which is proposed to be mediated by G_i (Maudsley et al., 2004). HEK293 cells stably-expressing the chemokine CCR5 receptor, which is an established G_i –coupled receptor (Mueller et al., 2002), were generated in order to serve as a positive control and cell surface expression of CCR5 was determined and verified using radioligand binding assays (data not shown).

Membranes containing the GnRH or CCR5 receptors were used in the [^{35}S]GTPγS binding assay with rapid filtration. Due to the low sensitivity of this assay, only G_i activation is detected, as this G protein subtype is well-expressed and has a high rate of nucleotide exchange (Harrison and Traynor, 2003). Basal [^{35}S]GTPγS binding curves, in the presence of varied concentrations of $MgCl_2$, were biphasic at both the GnRH receptor and CCR5-containing membranes (Fig.3.2), consistent with previous reports (Harrison and Traynor, 2003). Furthermore, the levels of basal binding at the two sets of membranes were similar, indicating that comparable levels of membrane proteins were present in the assay. In the presence of the CCR5 agonist, MIP-1β, a significant increase ($p<0.01$) in [^{35}S]GTPγS binding relative to basal levels was observed at 5, 10, 20 and 50mM $MgCl_2$ (Fig.3.2A). This result demonstrates that this assay is able to detect GPCR-induced activation of G_i and is thus functional. In contrast, the GnRH receptor-containing membranes did not exhibit an agonist-stimulated increase in [^{35}S]GTPγS binding at any of the investigated $MgCl_2$ concentrations (Fig.3.2B). Furthermore,

investigation of other optimising factors, discussed above, also failed to enable detection of GnRH receptor-G_i coupling (data not shown). This result thus suggests that the GnRH receptor is not able to induce robust activation of G_i.

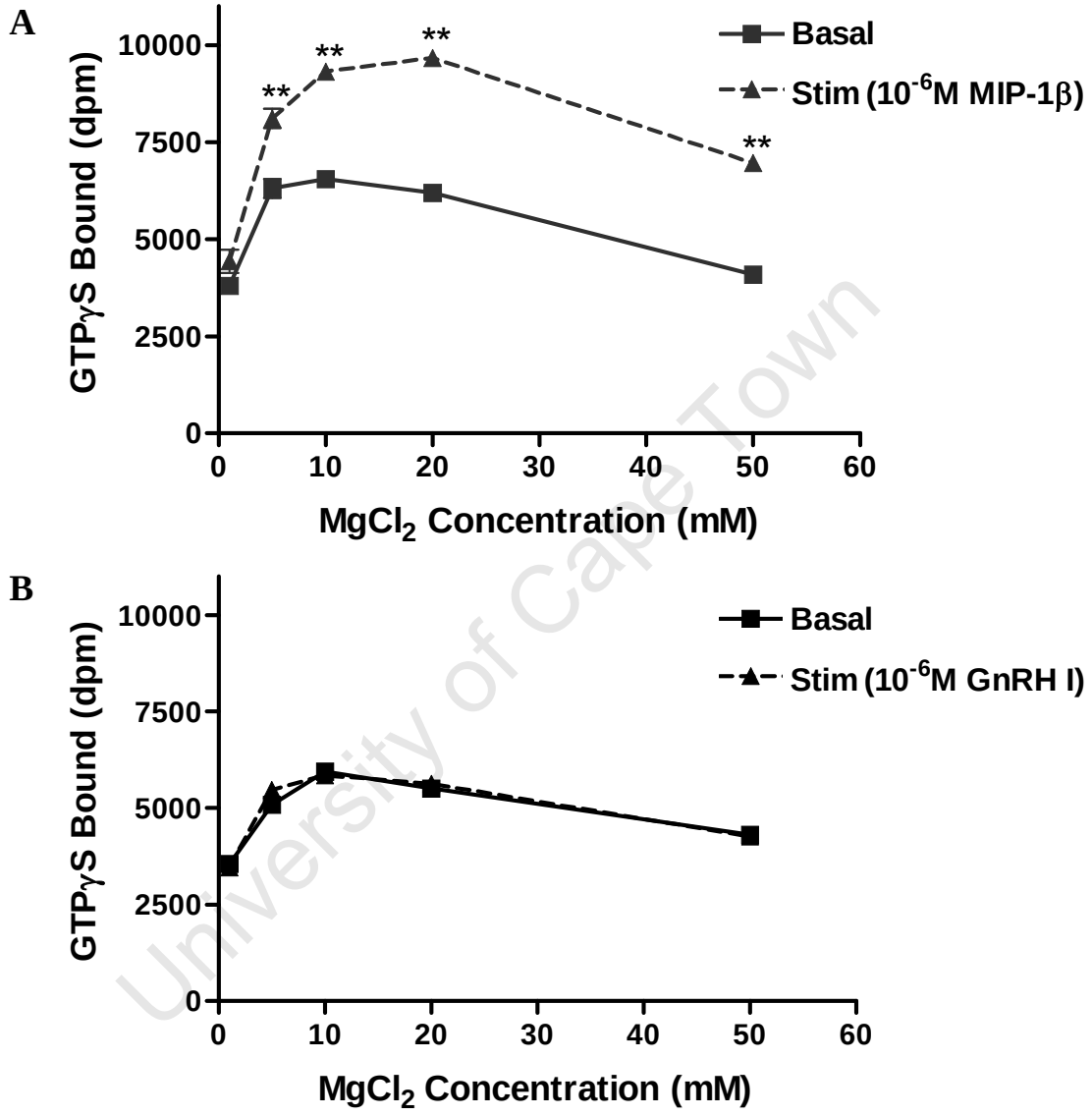


Figure 3.2. The [^{35}S]GTPγS Assay with rapid filtration in the presence of varied $MgCl_2$ concentrations measuring receptor-stimulated G_i activation. The assay was performed with HEK293 cells stably expressing the CCR5 receptor (A) or the GnRH receptor (B). Membranes were incubated in the presence (Stim) or absence (Basal) of the indicated ligand for 30 minutes prior to addition of the reaction mix containing [^{35}S]GTPγS. This step was included to allow the ligand-receptor interaction to reach equilibrium. Thereafter, membranes were incubated in a buffer containing 50mM HEPES (pH 7.4), 1mM EDTA, 0.1%BSA, 100mM NaCl, 10μM GDP, 0.2nM [^{35}S]GTPγS and varied $MgCl_2$ concentrations at 30^{o}C for 30 minutes. G protein-bound [^{35}S]GTPγS was collected by filtration through GF/C filters as described in the experimental procedures. Data are the mean ± S.E. of triplicate points. This figure is representative of numerous similar experiments investigating various optimising factors of the assay. **, $p<0.01$, Students *t* test, significantly different from basal values.

3.4.2 Investigation of GnRH receptor-G_i coupling using the Scintillation Proximity Assay (SPA)

As the GnRH receptor is classically a $G_{q/11}$-coupled receptor, its activation of G_i may not be robust and thus detection could require an assay with increased sensitivity. To this end, the SPA was performed. This assay has increased sensitivity, but also specificity, as it involves the use of G protein-subtype-specific antibodies (DeLapp et al., 1999). Here, membranes containing the GnRH receptor and the M_2 Muscarinic receptor (another well-established G_i-coupled GPCR) were used. Detection of [^{35}S]GTPγS bound by $G_{i1/2}$ proteins using SPA showed decreased basal [^{35}S]GTPγS binding in the presence of increasing concentrations of GDP, as expected (Fig.3.3A). The M_2 muscarinic receptor mediated a significant ($p<0.01$) agonist-induced increase in [^{35}S]GTPγS binding by $G_{i1/2}$ at all GDP concentrations (Fig.3.3A). The ability of the M_2 muscarinic receptor to facilitate $G_{i1/2}$ activation and thus GTPγS binding is verification of the functionality of the assay. However, the GnRH receptor did not show an agonist-induced increase in [^{35}S]GTPγS binding at any of the GDP concentrations investigated (Fig.3.3B). Further investigation of various optimising factors and using a G_{i3}-specific antibody also failed to detect GnRH receptor-G_i coupling (data not shown).

To confirm that the integrity of the GnRH receptor-containing membranes was intact, the ability of the GnRH receptor to couple to G_q was assessed. Using a G_q-specific antibody, basal and agonist-stimulated [^{35}S]GTPγS binding to G_q was determined (Fig.3.4A). In the presence of another GnRH receptor agonist, GnRH II, a significant ($p<0.01$) increase in [^{35}S]GTPγS binding to G_q was detected at 5, 10, 20 and 50mM $MgCl_2$ concentrations, relative to basal levels (Fig.3.4A). This result provides evidence that the membranes have functional GnRH receptors at sufficiently high levels to detect GnRH receptor-G protein coupling. The membranes prepared for this assay were also used for detection of G_i coupling, but GnRH II-elicited GnRH receptor-G_i coupling was not observed (Fig.3.4B), consistent with previous results obtained with GnRH I (Fig.3.3).

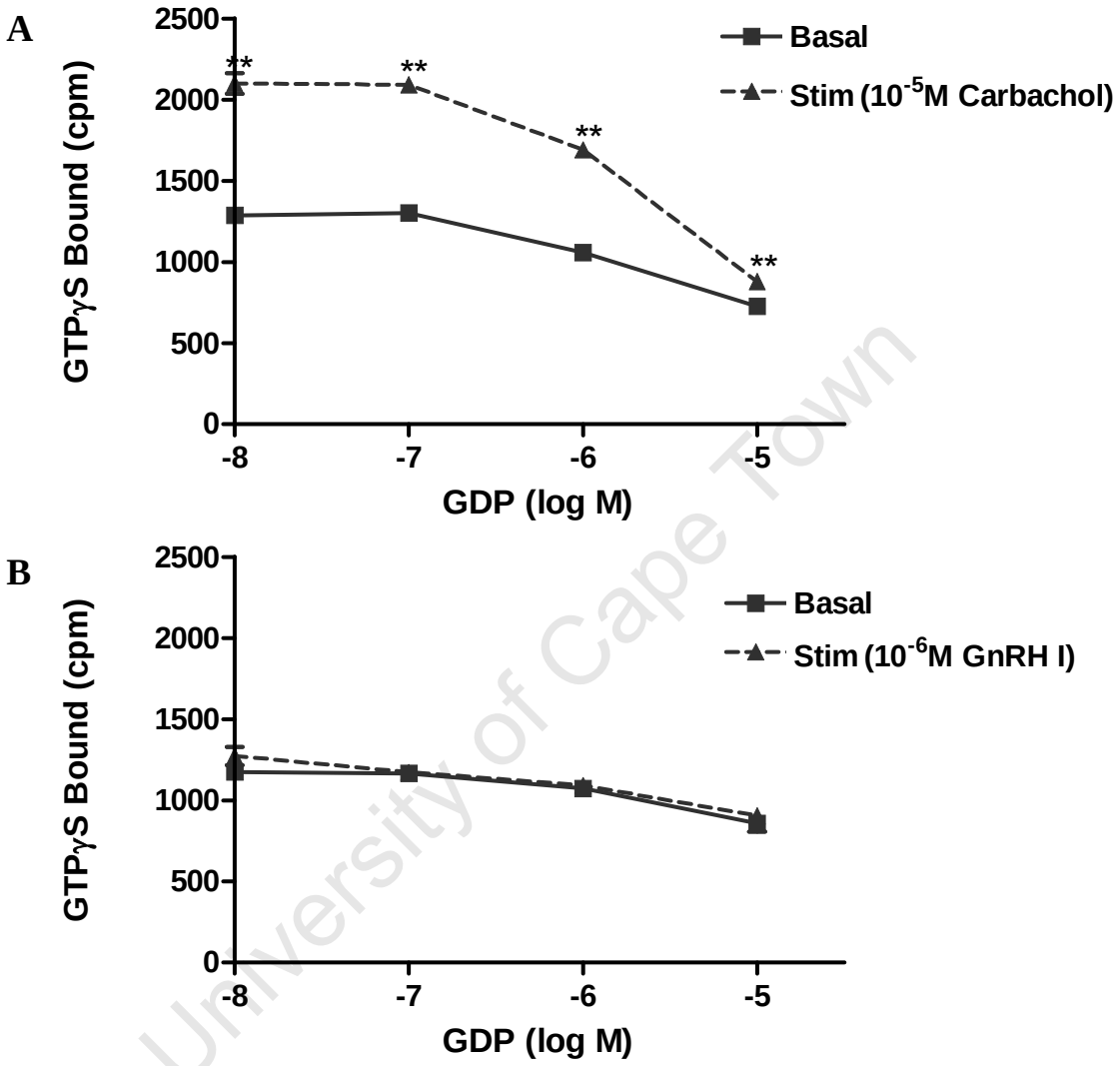


Figure 3.3. The Scintillation Proximity Assay with varying GDP concentrations measuring receptor-stimulated G_i activation. CHO and HEK293 cells stably expressing the M_2 Muscarinic receptor (A) or the GnRH receptor (B) respectively were incubated in the presence (Stim) or absence (Basal) of the indicated ligand for 60 minutes at 25°C. The assay buffer conditions included 20mM HEPES pH7.4, 5mM $MgCl_2$, 100mM NaCl, 0.2nM [^{35}S]GTPγS and varied GDP concentrations as indicated. Antibodies to $Gi_{1/2}$ subunits were added and anti-IgG-coated SPA beads facilitated capture of antibody-bound G proteins. $Gi_{1/2}$ proteins with radioactive [^{35}S]GTPγS bound stimulated the SPA beads to emit light, which was detected by liquid scintillation counting. Data are the mean ± S.E. of triplicate points. This figure is representative of numerous similar experiments investigating various optimising factors of the assay. **, $p<0.01$, Students *t* test, significantly different from basal values.

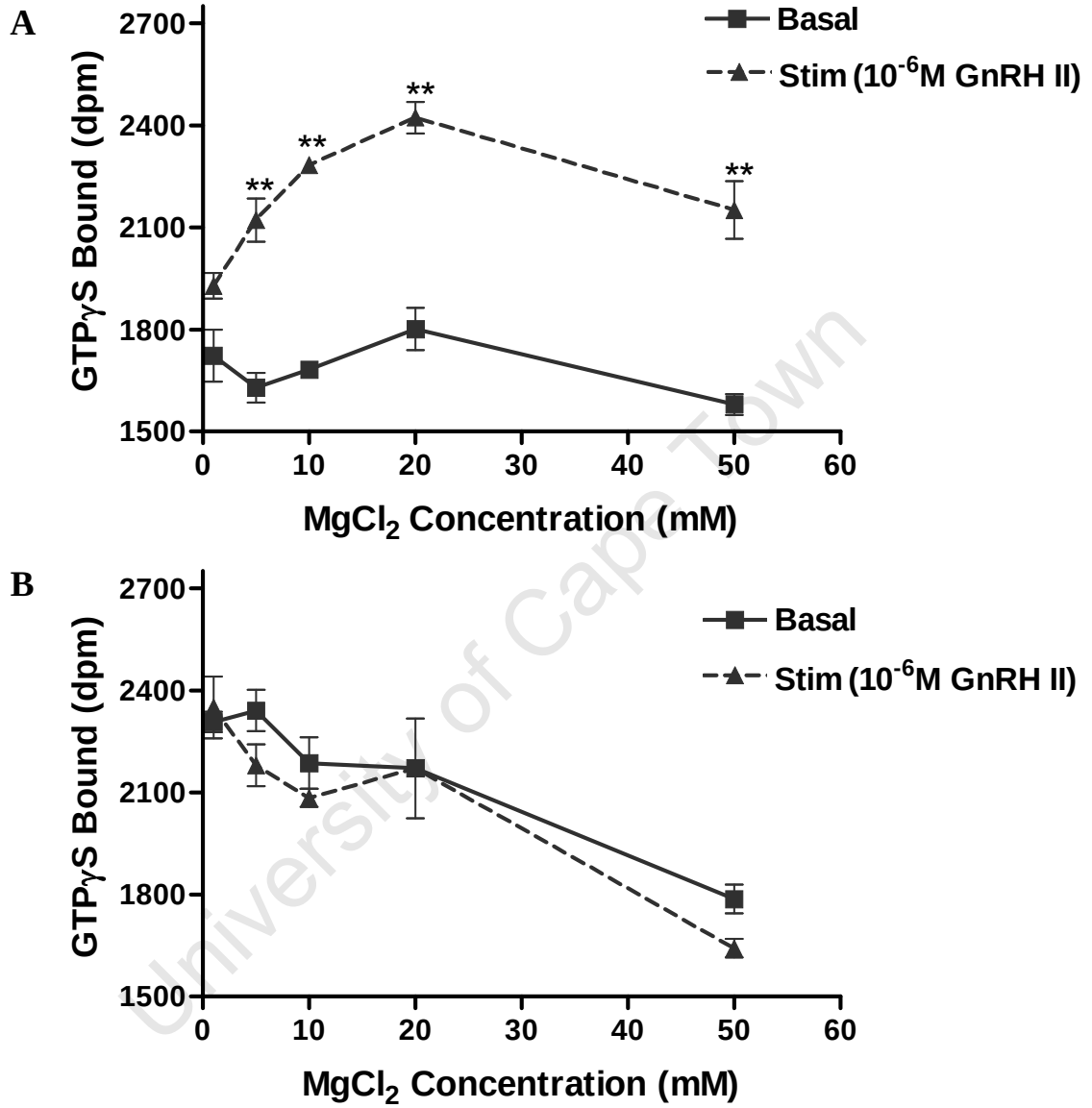


Figure 3.4. The Scintillation Proximity Assay with varying $MgCl_2$ concentrations measuring GnRH receptor-stimulated G_i and G_q activation. HEK293 cells stably expressing the GnRH receptor were incubated with the assay buffer (containing 20mM HEPES pH7.4; 100mM NaCl; 1.25mM [^{35}S]GTPγS and the indicated $MgCl_2$ concentrations) in the presence (Stim) or absence (Basal) of GnRH II for 60 minutes at 25 ^{o}C. G protein subtype-specific antibodies were then used to determine activation of G_q (A) and G_i (B) by the GnRH receptor. Data are the mean ± S.E. of triplicate points. This figure is representative of numerous similar experiments investigating various optimising factors of the assay. **, $p<0.01$, Students *t* test, significantly greater than basal values.

3.4.3 Investigation of GnRH receptor-G_i coupling using a reconstituted baculovirus-infected system

In a final attempt to detect GnRH receptor-G_i coupling, a baculovirus-infected reconstituted system enabling overexpression of both GnRH receptors and G_i proteins in insect cell membranes was developed for use in the [^{35}S]GTPγS binding assay. This system has successfully enabled expression of a large number of GPCRs, including the GnRH receptor, and has a number of advantages (Akermoun et al., 2005; Cheung and Hearn, 2003; Delahaye et al., 1997; Grunewald et al., 1996; McIntire et al., 2002; Neill et al., 1997). Firstly, high levels of functional GnRH receptor and G_i proteins can be achieved in a background of the low endogeneous G proteins expressed by the insect cells (Windh and Manning, 2002). Furthermore, alterations in the ratio of receptor:G protein is easily achieved by changing the multiplicity of infection (MOI) of the constructed viruses. By manipulating the system thus, the detection of even small signals would be achieved. Furthermore, G_i would not have to compete with G_q for coupling to the GnRH receptor.

Baculoviruses, enabling expression of the GnRH and CCR5 receptors and various Gα and Gβγ protein subunits, were constructed. Radioligand binding assays confirmed that the GnRH and CCR5 receptors were functionally expressed in this system (data not shown). Furthermore, using the CCR5 receptor as a positive control, the functionality of the G protein subunits was demonstrated (Fig.3.5A). A negative control of membranes containing the CCR5 receptor alone did not exhibit agonist-induced [^{35}S]GTPγS binding, as expected (Fig.3.5A). With the addition of $G\alpha_{i2}$, basal [^{35}S]GTPγS binding was increased ($p<0.01$) showing that the $G\alpha_{i2}$ protein was able to bind the GTP analogue. Additionally, in these membranes containing CCR5 and $G\alpha_{i2}$, MIP-1β induced a small, but significant ($p<0.01$), increase in [^{35}S]GTPγS binding, relative to the basal levels (Fig.3.5A). This result shows the ability of CCR5 to mediate activation of $G\alpha_{i2}$ and indicates that both CCR5 and the Gα subunit are functional. In membranes containing CCR5, $G\alpha_{i2}$ and $G\beta_1\gamma_2$, a large statistically significant increase ($p<0.01$) in

basal [^{35}S]GTPγS binding was observed. Gβγ subunits promote association of the Gα protein with the receptor. Thus the increased association of Gα with CCR5 and the known constitutive activity of CCR5 (Arias et al., 2003) are likely to be responsible for this increased signal. This provides evidence to support the functionality of the Gβγ subunits. Additionally, a further small, but significant ($p<0.01$), increase in [^{35}S]GTPγS binding was observed in response to agonist in these membranes, showing that this system enables detection of functional agonist-induced GPCR-mediated G protein coupling (Fig.3.5A). It is likely that the signal-to-noise ratio of this response would be improved by optimisation of assay conditions (such as increasing the Na^+ and GDP concentrations), but CCR5 was not the focus of this study and was thus not pursued. Extensive investigation of various optimising factors and assay conditions was explored for the GnRH receptor, but failed to detect GnRH receptor-G_i coupling. Fig.3.5B is a representative figure of these experiments showing that no GnRH receptor coupling to any of the G_i proteins was observed.

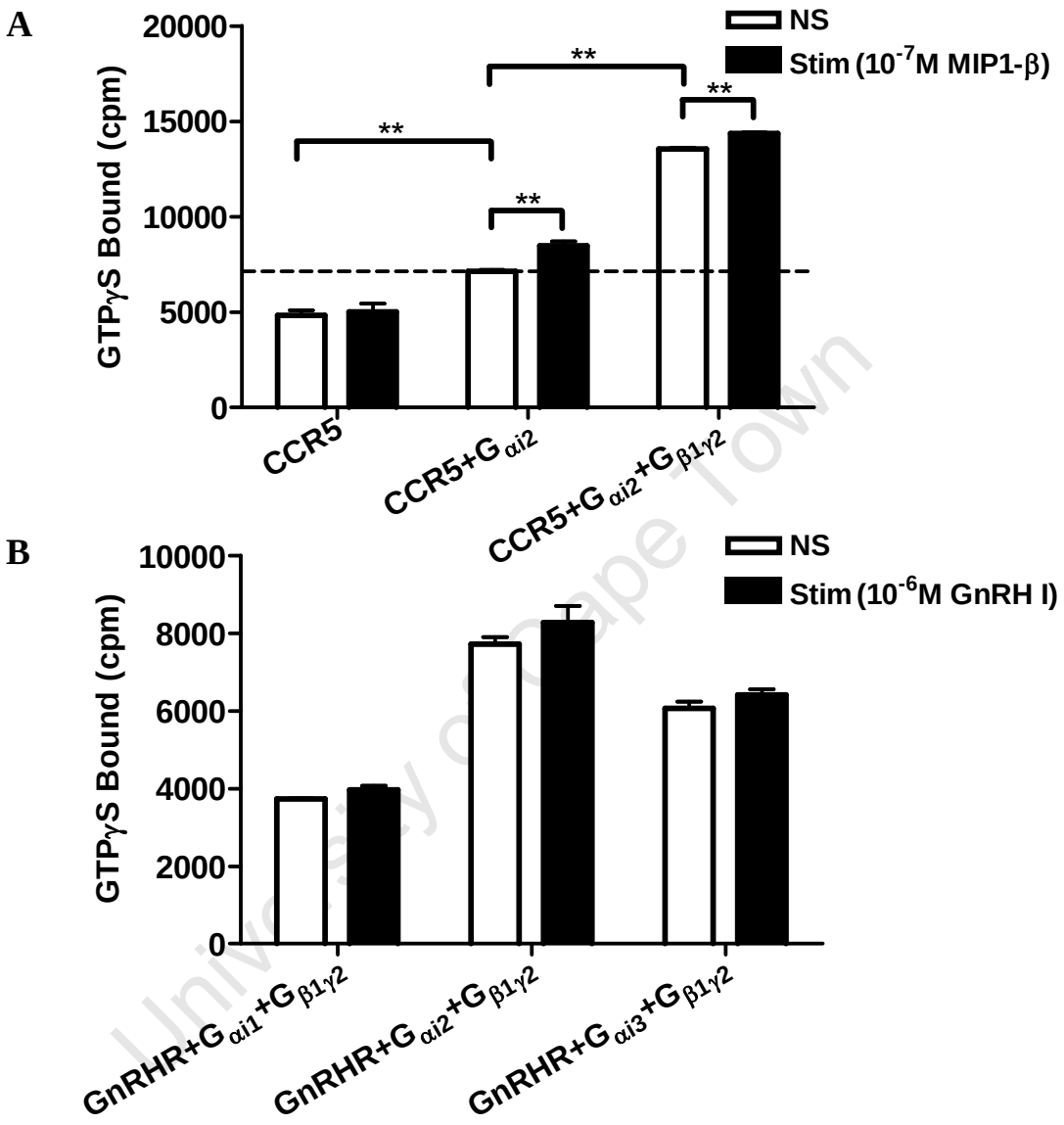


Figure 3.5. The [^{35}S]GTPγS Assay with rapid filtration with baculovirus-infected cell membranes. Sf21 cells were infected with baculoviruses inducing expression of the CCR5 (A) and GnRH receptors (B) and the indicated G protein subunits. The cells were infected at an MOI of 3:6:6 for receptor:Gα:Gβγ. Forty-eight hours following infection, membranes were harvested and the GTPγS assay with rapid filtration was performed as described in the experimental procedures. The assay conditions for the reaction included 20mM HEPES pH7.4, 100mM NaCl, 5mM $MgCl_2$ and 1μM GDP for 30 minutes at 27 ^{o}C in the presence or absence (NS, non-stimulated) of the indicated ligands. Data are the mean ± S.E. of triplicate points. This figure is representative of numerous similar experiments investigating various optimising factors of the assay. **, p<0.01, Students *t* test, significantly different from basal or bracketed values.

3.5 Discussion

Classical pituitary GnRH receptor signalling involves $G_{q/11}$ coupling (Pawson and McNeilly, 2005). However, the anti-proliferative effects of this receptor on cancer cells are suggested to be mediated by alternative coupling of the GnRH receptor to G_i proteins (Grundker and Emons, 2003; Grundker et al., 2001; Limonta et al., 1999; Millar et al., 2004; Millar et al., 2008). The suggestion that the anti-proliferative effects of the GnRH receptor are mediated by an alternative pathway to classical $G_{q/11}$ signalling is supported by the ability of classical antagonists to activate this pathway, as well as the reversal of efficacy of GnRH I and GnRH II at these pathways (Grundker and Emons, 2003; Millar et al., 2008; Yano et al., 1994). Thus, it has been proposed that different GnRH ligands stabilise distinct active conformations of the GnRH receptor and thus differ in their ability to activate $G_{q/11}$ and G_i (Maudsley et al., 2004; Millar et al., 2004). Within this framework, ligands which preferentially stabilise the G_i-coupled GnRH receptor conformation are proposed to facilitate the anti-proliferative effects of the receptor.

Nevertheless, the ability of the GnRH receptor to directly couple to G_i is disputed (Grosse et al., 2000) and has not been verified experimentally. In the present study, the ability of the GnRH receptor to directly couple to G_i was investigated using the [^{35}S]GTPγS binding assay. The data presented here show that GnRH receptor-G_i coupling could not be detected in any of the [^{35}S]GTPγS binding assays performed. Indeed, even in a reconstituted environment of high concentrations of the GnRH receptor and G_i proteins, coupling was not observed (Fig.3.5). In contrast, for the well-established G_i-coupled GPCRs, CCR5 and the M_2 muscarinic receptor, agonist-stimulated G_i activation was detected, thus confirming the functionality of the assay systems. This result shows that the GnRH receptor is not able to couple directly to the G_i family of G proteins.

The inability of the GnRH receptor to couple directly to G_i is supported by a previous study which reported exclusive GnRH receptor-mediated labelling of $G_{q/11}$ with [α-^{32}P]GTP azidoanilide and not other G protein subtypes (Grosse et al., 2000). However,

these results contradict other reports proposing GnRH receptor-G_i coupling. Some conclusions of G_i coupling resulted from experimental observations of GnRH receptor-induced inhibition of cAMP accumulation (Krsmanovic et al., 2003; Maudsley et al., 2004). Thus, an alternative explanation for these data is that Ca^{2+}, generated downstream of $G_{q/11}$ activation, facilitates inhibition of certain adenylate cyclase isoforms (Birnbaumer, 2007). However, some studies have specifically implicated GnRH receptor-mediated G_i activation by showing inhibition of GnRH receptor signalling with pertussis toxin (Imai et al., 2006; Kimura et al., 1999; Sim et al., 1995), G_i palmitoylation and release from the membrane in response to GnRH analogues (Krsmanovic et al., 2003; Stanislaus et al., 1998) and protection of pertussis toxin-mediated ADP ribosylation of G_i in the presence of GnRH analogues (Grundker et al., 2001; Imai et al., 1996). For these results to be consistent with the data presented in this chapter, these signalling events must occur downstream of initial receptor-mediated events. There are several mechanisms whereby this could occur. One suggestion is that GnRH receptor-G_i coupling occurs following a phosphorylation event which enables a switch in G protein coupling. Indeed, PKA-mediated phosphorylation of the β_2-adrenergic receptor (Daaka et al., 1997; Zamah et al., 2002) and prostacyclin receptor (Lawler et al., 2001), downstream of G_s activation, was required to observe G_i activation by these receptors. A second suggestion follows the observation of G_i coupling to RTKs (Delcourt et al., 2007; Kreuzer et al., 2004). It follows that GnRH receptor-mediated RTK transactivation could be responsible for the downstream effects on G_i (Kraus et al., 2001; Shah et al., 2003). Thus, it is proposed that the involvement of G_i in GnRH receptor signalling occurs downstream of initial receptor-mediated events. The demonstration that high agonist concentrations are required to facilitate G_i signalling mediated by the GnRH receptor further supports the conclusion that GnRH receptor-G_i coupling is not a proximal signalling event (Krsmanovic et al., 2003).

In conclusion, the experimental results presented here, show that the GnRH receptor is not able to couple directly to G_i. This result is significant as it negates the proposal that the proximal signalling event initiating the anti-proliferative effects of the GnRH receptor occurs via G_i. As the anti-proliferative signalling mediated by the GnRH

receptor does not require $G_{q/11}$ activation, this suggests that an alternative pathway is responsible. In light of the therapeutic implications for the treatment of reproductive cancers, future work is required to determine the signalling component(s) mediating this effect.

4 Chapter 4: Investigation of the coupling of the GnRH receptor to the SH2 domain-containing phosphatase 2, SHP-2

4.1 Abstract

GnRH receptor activation mediates anti-proliferative effects, associated with an increase in PTP activity, in cancer cells. In the present study, the ability of the GnRH receptor to activate the PTPs, SHP-1 and SHP-2 was explored. While unable to detect SHP-1 phosphorylation, GnRH I elicited a robust 2-fold increase in SHP-2 phosphorylation on Tyr^{542} in a time- and dose-dependent manner, illustrating specific and potent (EC_{50} = ~1nM) activation of this phosphatase. SHP-2 phosphorylation was detected in the presence of EGFR, G_q and G_i inhibitors, as well as excess Gβγ subunits, indicating that this pathway is activated independently of G proteins and EGFR transactivation. Furthermore, $G_{q/11}$-uncoupled $Tyr^{7.53}$Ala and $Asp^{7.49}$Ala GnRH receptors induced SHP-2 activation, as did a classical GnRH receptor antagonist, Ant135-18, supporting the proposal that distinct GnRH receptor conformations mediate SHP-2 and $G_{q/11}$ activation. In view of the G protein-independence of SHP-2 activation, the GnRH receptor sequence was analysed to determine if a direct SHP-2 binding motif could be identified. Analysis and immunoprecipitation experiments enabled identification of a canonical SH2-binding motif, pYxxL, at the bottom of TM7 of the GnRH receptor, which is constitutively phosphorylated and proposed to facilitate SHP-2 activation. GnRH I-elicited SHP-2 phosphorylation is mediated by src, as the src inhibitor, PP2, prevented the phosphorylation event. Furthermore, immunoprecipitation experiments reveal that src forms a direct binding complex with the GnRH receptor. A SHP-2 dominant negative construct, SHP-2(c/s) enabled identification of the contribution of SHP-2 signalling to GnRH I-elicited ERK and Akt regulation and thus this pathway may be associated with the anti-proliferative effects induced by the GnRH receptor.

4.2 Introduction

The GnRH receptor is central to mammalian reproduction (Pawson and McNeilly, 2005). In the pituitary, classical GnRH receptor signalling involves activation of the $G_{q/11}$ family of G proteins and consequent LH and FSH release, which, in turn, regulate gametogenesis and steroidogenesis at the gonads (Pawson and McNeilly, 2005). In contrast, GnRH receptors expressed in peripheral reproductive cancers have been shown to induce anti-proliferative signalling (Grundker and Emons, 2003; Grundker et al., 2001; Kraus et al., 2004; Maiti et al., 2005; Maudsley et al., 2004).

The endogenous GnRHs, GnRH I and GnRH II exhibit a reversal of potency at these two pathways. GnRH I has higher potency for $G_{q/11}$ signalling, while GnRH II is more potent at the anti-proliferative pathway (Grundker and Emons, 2003; Millar et al., 2008). In addition, certain GnRH receptor antagonists, classified due to their inability to facilitate $G_{q/11}$ activation, are able to induce anti-proliferative signalling at GnRH receptor (Maudsley et al., 2004; Yano et al., 1994). These data support the proposal that $G_{q/11}$ and anti-proliferative signalling at the GnRH receptor occur via distinct signalling pathways.

Efforts to identify the proximal events of the $G_{q/11}$-independent anti-proliferative arm of GnRH receptor signalling have suggested that the GnRH receptor couples to a second G protein family, the G_i proteins (Grundker et al., 2001; Imai et al., 2006; Imai et al., 1996b). However, our previous work (see chapter 3) and the work of others (Grosse et al., 2000) show that the GnRH receptor is unable to directly couple to G_i, suggesting that, if G_i is involved, its participation is downstream of other receptor-mediated events.

Emerging experimental evidence supports the ability of GPCRs to interact directly with src PTKs and/or SHP PTPs (Cao et al., 2000; Duchene et al., 2002; Fan et al., 2001; Ferjoux et al., 2003; Lopez et al., 1997; Sun et al., 2007a; Vatinel et al., 2006), facilitating coupling of the GPCR to phosphotyrosine signalling cascades. Interestingly, the GnRH receptor has been shown to induce PTP activity in peripheral cancer cells and furthermore, antagonises the proliferative signalling of the EGF and IGF-I receptors in certain cell types (Grundker et al., 2001; Imai et al., 1996a; Imai et al., 1996b; Marelli et al., 1999; Moretti et al., 1996). These signalling events are proposed to underlie the anti-proliferative effects of the GnRH receptor, therefore providing the impetus to investigate GnRH receptor-elicited PTP activity further. In this chapter, I sought to determine whether the GnRH receptor was able to activate the SH2 domain-containing phosphatases, SHP-1 and SHP-2 and whether this pathway could be activated independently of $G_{q/11}$ signalling in human breast cancer MCF-7 cell lines.

4.3 Experimental procedures

4.3.1 Materials

The src kinase specific inhibitor PP2, the PDGF receptor inhibitor AG1296, the EGF receptor inhibitor AG1478 and the SHP-2 inhibitor, NSC-87877 were all obtained from Calbiochem. Pertussis toxin was obtained from Sigma. The $G_{q/11}$ inhibitor, YM254890, was generously donated by Dr. Jun Takasaki. The cDNAs for SHP-2 (wild-type) (#12283) and the SHP-2 dominant negative construct SHP-2(c/s) (#12284) were acquired from Addgene (www.addgene.org) (Kolli et al., 2004). GnRH I and GnRH II were purchased from Sigma and Bachem respectively. The antagonists Antagonist 135-25, Antagonist 135-18 and Cetorelix were synthesised in our laboratory as described previously (Mamputha et al., 2007). cDNAs of the wildtype and mutant receptors were constructed previously (see below) and the vector control DNA was pcDNA3.1 (Invitrogen).

4.3.2 Cell culture and transfection

MCF-7 cells (obtained from ATCC) were transiently transfected with 15μg of DNA in a 0.4cm cuvette (containing $1.5X10^7$ cells per 0.7ml of Optimem Media (Gibco)) using electroporation. Cells were pulsed at 320V and 960 μF using a Bio-Rad Gene Pulser. After transfection, cells were plated into 10cm dishes and grown in DMEM (Gibco) supplemented with 10% fetal calf serum, 2% glutamine, 1% penicillin (10 000units/ml)/streptomycin (10 000mg/ml) at 37^oC in a humidified 5% CO_2 atmosphere. MCF-7 cells exhibit a high level of transfection efficiency following electroporation, which is demonstrated by the high levels of receptor expression that are observed with radioligand binding assays (Fig.S1).

Cell stimulations were performed 48h following transfection. Sixteen hours prior to stimulations, cells were incubated in serum-free media (DMEM, 2% glutamine, 1% penicillin/streptomycin, 10mM HEPES). Agonist stimulations were performed in serum-free media following preincubation with chemical inhibitors as described in the figure legends.

4.3.3 Immunoprecipitation and immunoblotting

Following ligand stimulation, cells were placed on ice and washed with ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS). The cells were then incubated with a NP-40-based Lysis Buffer (250mM NaCl, 50mM HEPES, 0.5% NP-40, 10% glycerol, 2mM EDTA, pH 8.0, supplemented with 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride and 1μg/ml leupeptin). Solubilised lysates were sonicated for 5 seconds and clarified by centrifugation at 20 000*g* for 15 min at which point the lysate was either mixed with an equal volume of 2XLaemli Buffer or subjected to overnight immunoprecipitation at 4^{o}C with constant agitation.

For immunoprecipitation of the HA-tagged GnRH receptors, 2μg of mouse anti-HA 12CA5 monoclonal antibody (Roche Applied Science) and 25μl of a 30% protein A/protein G agarose preconjugate slurry (Calbiochem) were incubated with cell lysates as described above. Immunoprecipitation of tyrosine phosphoproteins was achieved by incubating the lysate with 20μl of a 50% anti-PY20-agarose preconjugate slurry (Santa Cruz Biotechnology). Endogeneous src was immunoprecipitated with 20μl of a 50% preconjugated anti-src antibody slurry (c-src(N-16); Santa Cruz Biotechnology). Immune complexes were collected with a 5 min centrifugation step at 20 000X*g* and washed twice with the NP-40-based lysis buffer described above. After washing, 20μl of 2XLaemli Buffer was added to the collected immune complex pellet. The samples were heated to 95^{o}C for 5 min prior to loading onto the gels.

Samples were run by SDS-PAGE on 4-20% Tris-Glycine gels (Invitrogen) and transferred to Immobilon-FL 0.45μm polyvinylidene difluoride membranes (Millipore) using a Biorad Semi-dry transfer apparatus. Membranes were blocked with Odyssey Blocking buffer (Li-Cor Biosciences).

For immunodetection of relevant proteins, the following antibodies were used. To detect phosphorylated SHP-1, anti-SHP-1 (pTyr^{536}) (ECM Biosciences) was used at a 1:500 dilution. Anti-SHP-2 (pTyr^{452}) (Calbiochem) was used to detect phosphorylated SHP-2 at a 1:1000 dilution. The anti-HA 12CA5 monoclonal antibody (Roche Applied Science) was used to detect HA-tagged GnRH receptors at

1:500 dilution. Activation of src was detected using a Tyr^{416} phospho-specific src antibody (Cell signalling). pERK (phospho-p44/42 MAP Kinase (Thr^{202}/Tyr^{204}) rabbit antibody), ERK (p42 MAP Kinase (3A7) mouse monoclonal antibody) and pAkt (Phospho (Ser^{473}) rabbit monoclonal antibody) were all obtained from Cell Signalling and used at 1:1000 dilution. Primary antibodies were detected with Goat IRDye800 conjugated anti-mouse antibodies (Rockland) and Goat Alexa Fluor 680/700 conjugated anti-rabbit antibodies (Invitrogen Molecular Probes) at a 1:5000 dilution for all primaries with the exception of ERK and pERK which was a 1:10 000 dilution. The membranes were visualised and quantified using the Odyssey Li-Cor infrared imaging system and application software version 2.1.12.

4.3.4 Data analysis

Data were plotted and analysed using Graphpad Prism 4.0 (Graphpad Software Inc.). Sigmoidal dose-response curves were fitted to the relevant data and the EC_{50} value determined. Statistical analysis was performed using a Students *t* test or a one-way ANOVA with a Dunnett's multiple comparison test.

4.4 Results

4.4.1 Determination of GnRH I-elicited SHP-1 and SHP-2 activation

In the inactive state, SHP-1 and SHP-2 exist in an autoinhibited conformation where the N-terminal SH2 domain folds back against the phosphatase domain and inhibits its activity (Neel et al., 2003). Phosphorylation of SHP-1 and SHP-2 on Tyr^{536} and Tyr^{542} in their C-terminal tails, respectively, provides a site for engagement of the N-terminal SH2 domain of the SHP, relieving the inhibition of the phosphatase and enabling its activation (Neel et al., 2003; Poole and Jones, 2005). Thus, in order to investigate whether the human GnRH receptor activates SHP-1 and SHP-2, MCF-7 cells transiently expressing the GnRH receptor were stimulated with GnRH I and the phosphorylation status of these SHP Tyr residues assessed.

Stimulation of the transfected MCF-7 cells with GnRH I led to a robust statistically significant ($p<0.01$) 2-fold increase in SHP-2 phosphorylation on Tyr^{542} (Fig.4.1). The induction of SHP-2 phosphorylation in response to GnRH I was rapid, with detection of SHP-2 phosphorylation after only two minutes of stimulation. Maximal

GnRH I-elicited SHP-2 phosphorylation was achieved by 5 minutes and the phosphorylation status remained protracted over 60 minutes of GnRH I stimulation (Fig.4.1). Untransfected MCF-7 cells did not induce SHP-2 activation, indicating that this effect is specifically mediated by the human type I GnRH receptor (Fig.S2). Thus, in response to GnRH I, the GnRH receptor is able to induce robust and rapid activation of SHP-2 in a time-dependent manner in MCF-7 cells.

To assess the potency of GnRH I-elicited SHP-2 activation, transiently transfected MCF-7 cells were stimulated with various concentrations of GnRH I. GnRH I-elicited SHP-2 phosphorylation occurred in a dose-dependent manner (Fig.4.2). Analysis of the fitted dose-response curve of SHP-2 phosphorylation indicates that GnRH I had an EC_{50} value of ~1.0nM (Fig.4.2). This result shows that SHP-2 is specifically and potently phosphorylated in response to GnRH I. Interestingly, SHP-2 detection on western blots is a wide band (Fig.4.1), that consists of two bands (see Fig.4.2). These two bands may represent an extended linear conformation of the protein (which will run slower) and a folded conformation where the C terminal phosphorylated tyrosine is bound by the N-SH2 domain of the protein (Neel et al., 2003). Alternatively, they may represent SHP-2 forms with differential modifications (Poole and Jones, 2005).

In contrast to SHP-2, neither basal nor GnRH I-induced phosphorylation of SHP-1 on Tyr^{536} was detected (Fig.S3) despite reports that SHP-1 expression occurs in these cells (Thangaraju et al., 1999). Taken together, these results indicate that, while we were unable to detect GnRH I-induced phosphorylation of SHP-1 on Tyr^{536}, GnRH I elicits potent and robust phosphorylation of SHP-2 on Tyr^{542} (Fig.4.1 and Fig.4.2).

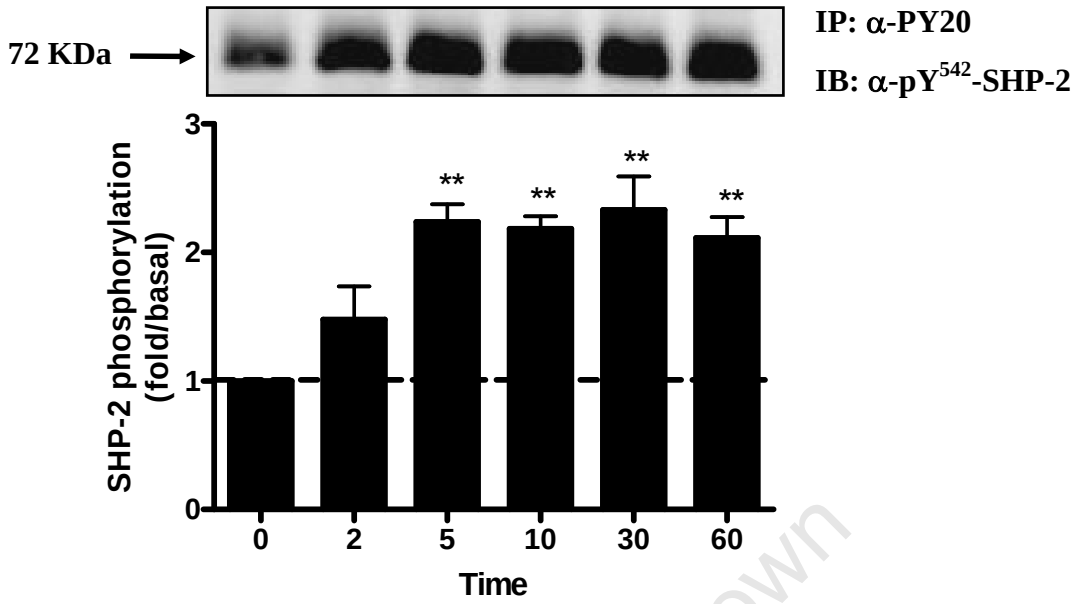


Figure 4.1. Time-course of GnRH I-elicited SHP-2 activation. Forty-eight hours following transient transfection with the human GnRH receptor and overnight serum starvation, cells were treated with 100nM GnRH I for the indicated time points. Phosphorylated proteins were immunoprecipitated with the PY20 antibody and SHP-2 phosphorylation on Tyr^{542} was detected. Data represent the mean ± S.E. of three independent experiments. **, $p<0.01$, significantly different from control (NS (non-stimulated) cells); one-way ANOVA with Dunnett's multiple comparison test.

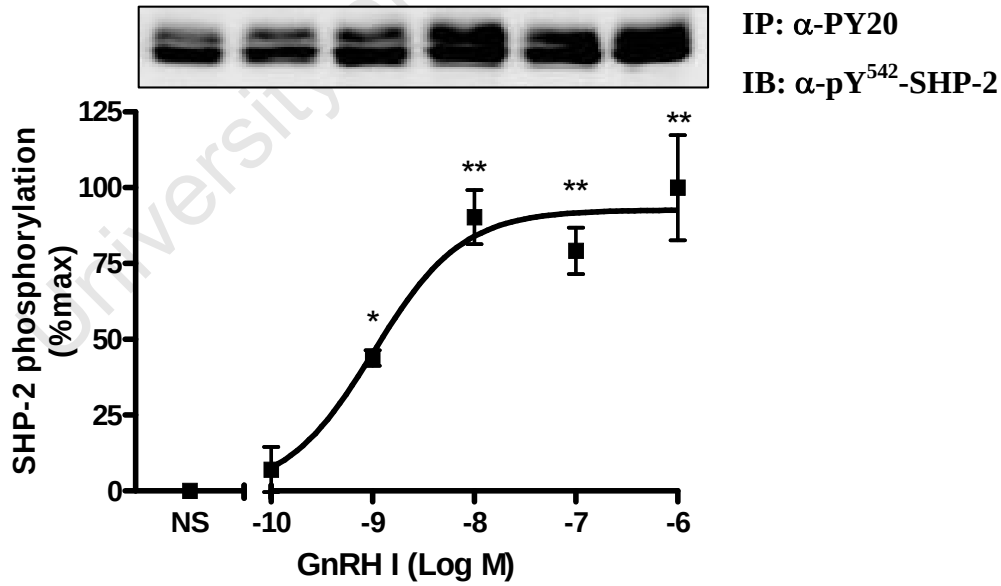


Figure 4.2. Dose-response curve of GnRH I-elicited SHP-2 phosphorylation. MCF-7 cells were prepared as described above, treated with various concentrations of GnRH I for 5 minutes and SHP-2 phosphorylation detected. A sigmoidal dose-response curve was fitted to the data and the EC_{50} for SHP-2 phosphorylation by GnRH I determined as ~1.0nM. Data represent the mean ± S.E. of three independent experiments. *, $p<0.05$, **, $p<0.01$, significantly different from control (NS cells); one-way ANOVA with Dunnett's multiple comparison test.

4.4.2 Effects of the EGFR inhibitor, AG1478, on GnRH I-elicited SHP-2 activation

Tyrosine kinase receptors, such as the EGF receptor, mediate SHP-2 activation (Araki et al., 2003; Bennett et al., 1994; Chong and Maiese, 2007; Wang et al., 2006). Thus, considering the ability of the GnRH receptor to induce transactivation of the EGFR in several cell lines (Dobkin-Bekman et al., 2006; Kraus et al., 2001; Shah et al., 2003), it was necessary to determine if this represents the mechanism for SHP-2 activation in response to GnRH I. To establish this, cells were preincubated with a specific inhibitor to the EGF receptor, AG1478, prior to GnRH I stimulation. The results show that GnRH I elicited statistically significant ($p<0.05$) SHP-2 activation in the presence of the EGFR inhibitor, comparable to the stimulation obtained in the absence of the inhibitor (Fig.4.3). This shows that GnRH I-elicited SHP-2 activation does not occur by transactivation of the EGFR and indicates that another pathway must be responsible.

4.4.3 The role of G protein signalling in GnRH I-elicited SHP-2 activation

The major pathway of GPCR signalling involves coupling to G proteins. Thus, an investigation of the effects of inhibition of this signalling pathway on GnRH I-elicited SHP-2 activation was performed. Classical GnRH receptor signalling involves activation of the $G_{q/11}$ family of G proteins (Kraus et al., 2001; Naor et al., 2000; Pawson and McNeilly, 2005). Additionally, GnRH receptor coupling to the G_i family of G proteins has been suggested to mediate GnRH-induced activation of PTP activity in ovarian cancer cells (Grundker et al., 2001). Thus to assess whether the G proteins, G_q or G_i, mediate the coupling of the GnRH receptor to SHP-2, specific inhibitors to the $G_{q/11}$ and G_i proteins, YM254890 and PTX respectively, were preincubated with the cells prior to GnRH I stimulation. Furthermore, overexpression of the $G\beta_1$ and $G\gamma_2$ subunits was performed to determine the effects of these G protein subunits on GnRH I-elicited SHP-2 activation.

In the presence of 100nM of the $G_{q/11}$ inhibitor, YM254890, which completely inhibits the GnRH receptor-mediated IP responses (Fig.S4), GnRH I induced statistically significant ($p<0.05$) phosphorylation of SHP-2 (Fig.4.4). This result shows that GnRH I-elicited SHP-2 activation can occur independently of the $G_{q/11}$

protein. Nevertheless, compared with control cells, SHP-2 phosphorylation in response to GnRH I was reduced in the presence of YM254890, showing that this inhibitor partially inhibits the phosphorylation event. In the presence of the G_i inhibitor, PTX, GnRH I elicited robust statistically significant ($p<0.05$) SHP-2 activation, comparable with control levels (Fig.4.4). This result shows that GnRH receptor-mediated SHP-2 phosphorylation does not occur via activation of the G_i family of G proteins. Following overexpression of the $G\beta_1\gamma_2$ subunits, which associate with and thereby inhibit the activity of the $G\alpha$ subunits, the GnRH receptor also induced statistically significant ($p<0.05$) phosphorylation of SHP-2 (Fig.4.4). This result suggests that GnRH I-elicited SHP-2 activation can occur independently of all G protein subtypes. However, compared with control cells, in the presence of excess $G\beta\gamma$ subunits GnRH I-elicited SHP-2 phosphorylation was partially inhibited, similar to the inhibition observed with YM254890. Taken together, these results suggest that SHP-2 activation in response to GnRH I exhibits partial $G_{q/11}$ protein dependence, but can also occur in a G protein-independent manner.

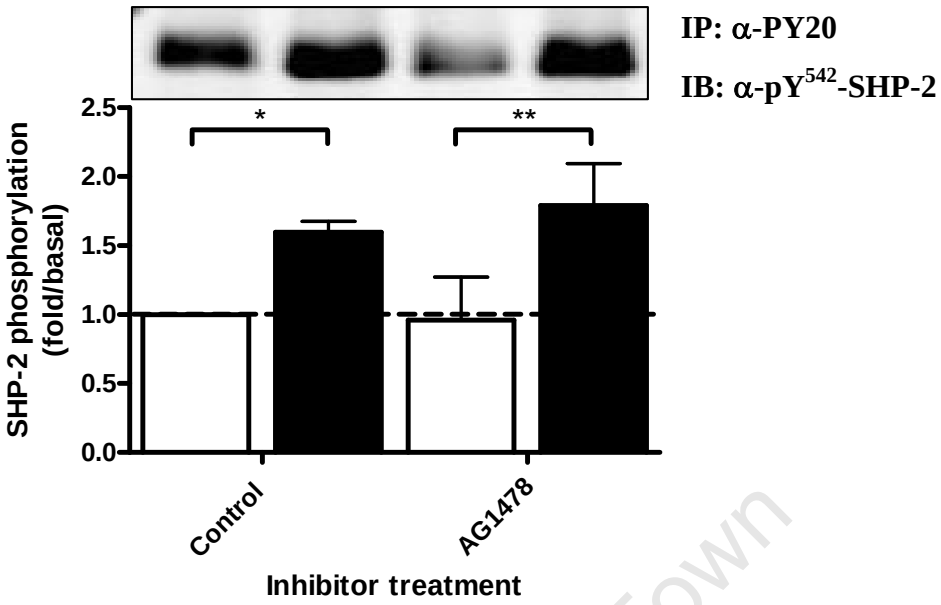


Figure 4.3. The effect of the EGFR inhibitor, AG1478, on GnRH I-elicited SHP-2 phosphorylation. Serum-starved transiently transfected MCF-7 cells expressing the GnRH receptor were incubated with DMSO (control) or with AG1478 (100nM) for 30 minutes prior to GnRH I stimulation. Cells were stimulated with 100nM GnRH I (filled bars) or with vehicle control (open bars) for 5 minutes. The data represent the mean ± S.E. of 2-3 independent experiments. *, $p<0.05$, **, $p<0.01$, significantly different from NS control; Students *t* test.

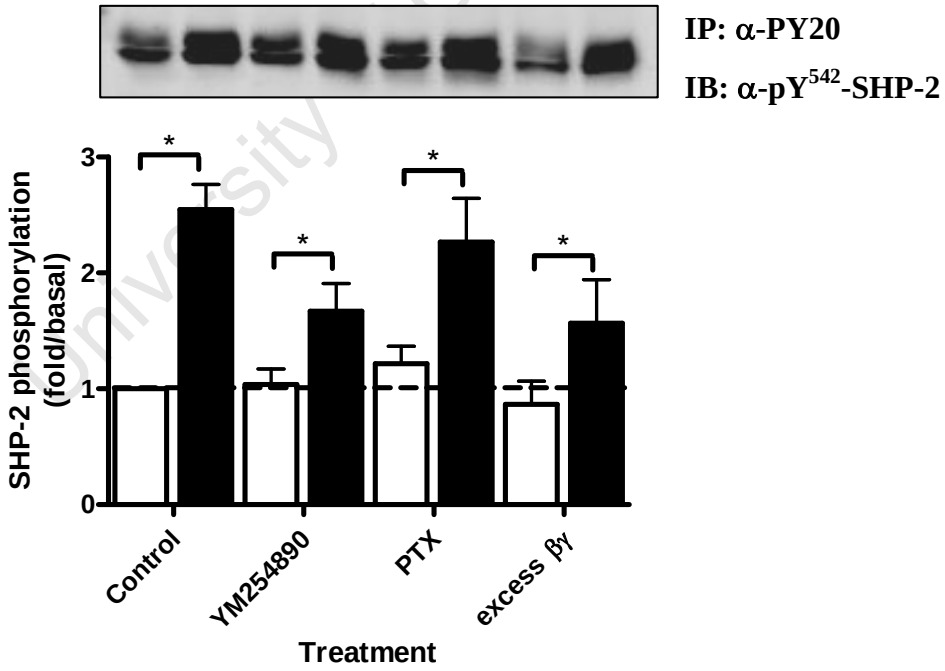


Figure 4.4. The effects of inhibitors to G_q and G_i and Gβγ overexpression on GnRH I-elicited SHP-2 phosphorylation. MCF-7 cells were transiently transfected with the GnRH receptor and a vector control (for control and inhibitor-treated cells) or $G\beta_1\gamma_2$ (equal concentrations of each) at a 1:1 ratio. Forty-eight hours following transfection and overnight serum-starvation, cells were treated with PTX (200ng/ml; 16 hours), YM254890 (100nM; 30 minutes) or DMSO as a control (30 minutes). Filled and open bars represent cells stimulated with 100nM GnRH I or vehicle control for 5 minutes respectively. The data represent the mean ± S.E. of at least three independent experiments. *, $p<0.05$, significantly different from NS control; Students *t* test.

4.4.4 Investigation of SHP-2 activation in response to GnRH II and three classical GnRH receptor antagonists

Considering that the above results suggest a G protein-independent mechanism of GnRH-induced SHP-2 activation, I was interested in determining if classical antagonists of GnRH receptor-mediated $G_{q/11}$ signalling could induce SHP-2 activation. Furthermore, the ability of the second endogenous GnRH receptor agonist, GnRH II, to facilitate SHP-2 activation at the GnRH receptor, was in question. Thus cells were stimulated, for 5 minutes, with GnRH I as a control, GnRH II and three GnRH receptor antagonists, Ant135-25, Cetorelix and Ant135-18, and the SHP-2 phosphorylation responses assessed. GnRH II induced robust statistically significant ($p<0.01$) SHP-2 phosphorylation, comparable with GnRH I-elicited SHP-2 phosphorylation (Fig.4.5). Thus, both the endogenous GnRH agonists, GnRH I and GnRH II, are able to mediate SHP-2 activation at the GnRH receptor. Evaluation of the ability of the selected antagonists to mediate SHP-2 activation revealed that Ant135-18 elicited statistically significant ($p<0.05$) SHP-2 phosphorylation of ~30% the level induced by GnRH I. This result shows that classical GnRH receptor antagonists can activate SHP-2 and supports the proposal that GnRH receptor-mediated SHP-2 activation can occur in a $G_{q/11}$-independent manner. Furthermore, it suggests that GnRH receptor conformational requirements that mediate SHP-2 activation differ from those that facilitate $G_{q/11}$ activation. In contrast, 5 minute stimulation with Ant135-25 and Cetorelix did not induce discernible SHP-2 phosphorylation, suggesting that these antagonists stabilise different receptor conformations to Ant135-18. In summary, these data support the proposal that the GnRH receptor is able to adopt multiple receptor conformations with distinct signalling profiles at the $G_{q/11}$ and SHP-2 signalling pathways.

4.4.5 Investigation of SHP-2 activation by $G_{q/11}$-uncoupled mutant GnRH receptors

In order to investigate the GnRH receptor conformational requirements for SHP-2 activation, the ability of three previously characterised GnRH receptor mutants, the $Ala^{6.29}Lys$, $Asp^{7.49}Ala$ and $Tyr^{7.53}Ala$ receptors, to facilitate SHP-2 phosphorylation was evaluated. Each of these mutations induces uncoupling of the GnRH receptor from $G_{q/11}$ signalling (Arora et al., 1996; Flanagan et al., 1999; Lu et al., 2005; Myburgh et al., 1998), but the mechanism whereby this is achieved differs. The

Ala$^{6.29}$Lys mutation occurs within the third intracellular loop, below TM6, and is thought to facilitate $G_{q/11}$ uncoupling by sterically hindering G protein association with the receptor (Myburgh et al., 1998). In contrast, the Asp$^{7.49}$Ala and Tyr$^{7.53}$Ala mutations occur in the TM domains of the receptor, specifically within the highly conserved D/NPxxY motif. These residues are proposed to make intramolecular interactions in both the inactive and active states of GPCRs (Fritze et al., 2003; Palczewski et al., 2000; Prioleau et al., 2002; Urizar et al., 2005). Thus, the Asp$^{7.49}$Ala and Tyr$^{7.53}$Ala mutations are suggested to induce $G_{q/11}$ uncoupling by preventing formation of intramolecular interactions that stabilise the active receptor conformation. Furthermore, mutation of both Asp$^{7.49}$ and Tyr$^{7.53}$ has been implicated in facilitating different conformational states of GPCRs and activation of non-G protein signalling pathways (Flanagan et al., 1999; Kalatskaya et al., 2004; Mitchell et al., 1998; Prioleau et al., 2002).

The Ala$^{6.29}$Lys mutant receptor was not able to induce SHP-2 phosphorylation in response to GnRH I (Fig.4.6). This result suggests that the steric constraint imposed by this mutation on $G_{q/11}$ protein coupling also hinders SHP-2 activation. Thus the molecular requirements for $G_{q/11}$ coupling and SHP-2 activation may overlap. In contrast, the Asp$^{7.49}$Ala mutation did not prevent SHP-2 phosphorylation and GnRH I-elicited SHP-2 activation at this receptor was statistically significant ($p<0.05$) (Fig.4.6). This result further supports the proposal that SHP-2 activation occurs independently of $G_{q/11}$ activation and that the conformational requirements for SHP-2 and $G_{q/11}$ activation are distinct. This is also supported by the ability of the Tyr$^{7.53}$Ala mutation to mediate statistically significant ($p<0.05$) SHP-2 phosphorylation comparable with wildtype receptor levels, despite being uncoupled from $G_{q/11}$ signalling. Interestingly, the Tyr$^{7.53}$Ala mutant receptor exhibited elevated basal SHP-2 phosphorylation relative to the wildtype receptor (Fig.4.6), suggesting that this mutation induces constitutive activity at the SHP-2 signalling pathway. This is consistent with previous reports suggesting that Tyr$^{7.53}$ induces selective constitutive activity in other GPCRs and that this residue represents a receptor conformational molecular switch (Kalatskaya et al., 2004; Prioleau et al., 2002). The ability of this mutation of the GnRH receptor to induce constitutive activity at the SHP-2 signalling pathway, but not at the $G_{q/11}$ protein pathway, further supports the proposal that the

active conformational requirements of the GnRH receptor differ for the two pathways. Thus, together, these data suggest that the molecular requirements of the GnRH receptor that facilitate $G_{q/11}$ and SHP-2 coupling overlap, but are governed by distinct receptor conformations.

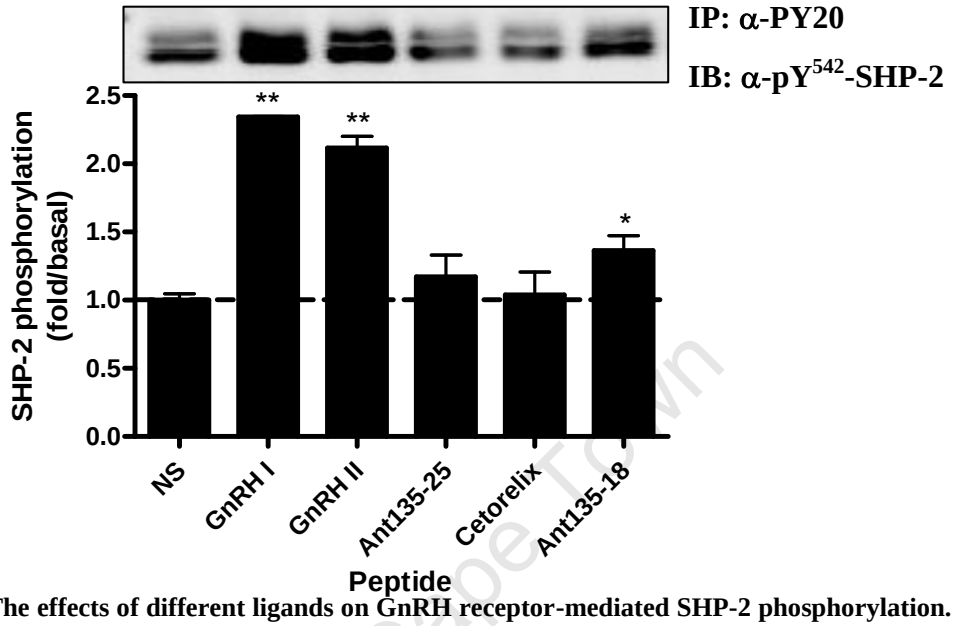


Figure 4.5. The effects of different ligands on GnRH receptor-mediated SHP-2 phosphorylation. MCF-7 cells transiently expressing the GnRH receptor (48 hrs) were serum-starved (overnight) and treated for 5 min with 100nM of GnRH I or GnRH II or 1μM of Ant135-25, Cetorelix or Ant135-18. NS cells were treated with vehicle control for 5 min. Data are the mean ± S.E. of three independent experiments. *, $p<0.05$, **, $p<0.01$, significantly different from NS control; Students *t* test.

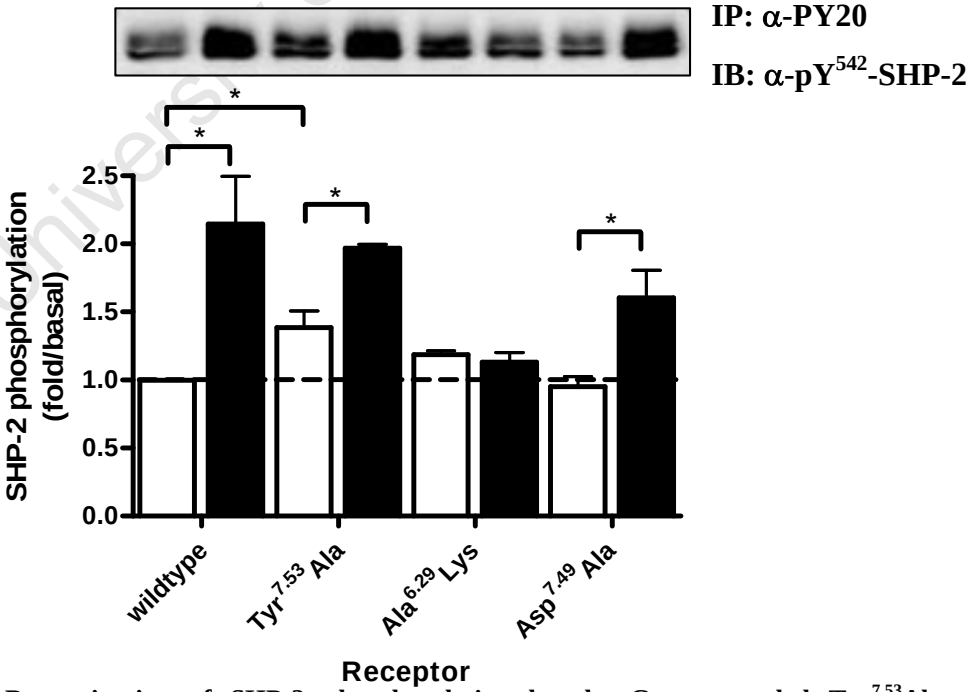


Figure 4.6. Determination of SHP-2 phosphorylation by the $G_{q/11}$-uncoupled $Tyr^{7.53}Ala$, $Ala^{6.29}Lys$ and $Asp^{7.49}Ala$ GnRH receptor mutants. The indicated receptors were transiently transfected into MCF-7 cells. Serum-starved cells were stimulated with 100nM GnRH I (filled bars) or with vehicle control (open bars) for 5 minutes. Data are the mean ± S.E. of 2-3 independent experiments. *, $p<0.05$, brackets indicate bars compared; Students *t* test.

4.4.6 GnRH receptor expression and tyrosine phosphorylation

The above experimental results provided clues for identification of a putative GnRH receptor motif that facilitates SHP-2 activation. Firstly, the ability of GnRH I-elicited SHP-2 activation to occur independently of G proteins suggests that SHP-2 may form a direct binding complex with the GnRH receptor. Secondly, the increased basal activity of the $Tyr^{7.53}$Ala receptor is illuminating as $Tyr^{7.53}$ interactions are proposed to alter the conformation of TM7 and the C-terminal tail of GPCRs (Weinstein, 2005). As the GnRH receptor lacks a C-terminal tail, this suggests that the motif important for SHP-2 activation may occur in TM7 of the GnRH receptor. Analysis of the amino acid sequence of the GnRH receptor reveals a putative SH2-binding domain, YxxL, located within the four terminal amino acids of TM7 of the GnRH receptor (Fig.4.7). Indeed, similar SH2-binding motifs have been identified in a few other GPCRs and were necessary to observe the activation of SHPs by these receptors (Duchene et al., 2002; Ferjoux et al., 2003; Vatinel et al., 2006). However, in order for this domain to represent a canonical SH2 binding site, the tyrosine residue within this motif must be phosphorylated (Pawson, 2004). Thus, it was necessary to investigate whether tyrosine phosphorylation of the GnRH receptor could be detected. The $Tyr^{7.53}$Ala mutation was included in these experiments as this residue, with the exception of the Tyr within the YxxL motif, is the only additional tyrosine accessible on the intracellular surface of the receptor (see Fig.4.7). Thus if Tyr phosphorylation of this mutant GnRH receptor could be detected, this would imply that the $Tyr^{7.55}$ of the YxxL motif is Tyr phosphorylated.

First, wildtype and $Tyr^{7.53}$Ala GnRH receptors, which contain HA-tags at their N-termini, were transiently transfected into MCF-7 cells and expression of the receptors evaluated. Four distinct GnRH receptor bands were detected (Fig.4.8), consistent with previous reports (Sedgley et al., 2006). Three of these bands were ~32, ~34 and ~36KDa in size. These bands are likely to represent GnRH receptors with differential modifications, like glycosylation and/or phosphorylation. The fourth band was larger and wider and was ~60-70kDa in size. This band may represent receptor dimers. The most intense GnRH receptor band was ~36KDa in size and thus represents the most predominant GnRH receptor form expressed in these cells. The $Tyr^{7.53}$Ala receptor

exhibited a similar distribution of GnRH receptor bands and had comparable receptor expression with the wildtype receptor (Fig.4.8).

Next, tyrosine phosphorylation of the GnRH receptors was determined (Fig.4.9). A distinct band of ~32KDa was detected following immunoprecipitation of tyrosine phosphorylated proteins and detection of the GnRH receptor HA tag. This band was not observed in untransfected MCF-7 cells indicating that this band is specific for the GnRH receptor (Fig.4.9). This shows that the wildtype GnRH receptor undergoes tyrosine phosphorylation. The absence of bands corresponding to other GnRH receptor isoforms (see Fig.4.8) suggests that the population of GnRH receptors are heterogeneous, with some receptors tyrosine phosphorylated, while others are not. Both the wildtype receptor and $Tyr^{7.53}$Ala receptor exhibited comparable tyrosine phosphorylation (Fig.4.9). This suggests that the GnRH receptor is tyrosine phosphorylated, but that the phosphorylation site is not $Tyr^{7.53}$. As the only additional Tyr accessible at the intracellular surface of the receptor is $Tyr^{7.55}$ of the YxxL motif, I propose that this is the tyrosine phosphorylation site of the receptor. Interestingly, tyrosine phosphorylation of the GnRH receptor was constitutive and increased only slightly following agonist stimulation (Fig.4.9). Indeed, time-course studies revealed very little change in the tyrosine phosphorylation status of the GnRH receptor even after stimulation for time points up to 60 minutes (Fig.S5).

These data suggest that the human GnRH receptor is constitutively phosphorylated on Tyr of the YxxL motif in TM7, suggesting that this motif represents a canonical pYxxL SH2-binding domain. This motif thus represents a putative mechanism for SHP-2 recruitment to the GnRH receptor independently of G proteins, which may facilitate SHP-2 phosphorylation in response to GnRH I. However, attempts to co-immunoprecipitate the GnRH receptor and SHP-2 (with and without agonist stimulation) to confirm a direct interaction between the two proteins were unsuccessful (data not shown). This suggests that SHP-2 interaction with the GnRH receptor may be dynamic, low affinity or alternatively, the antibodies in the immunoprecipitation experiments disrupted the interaction.

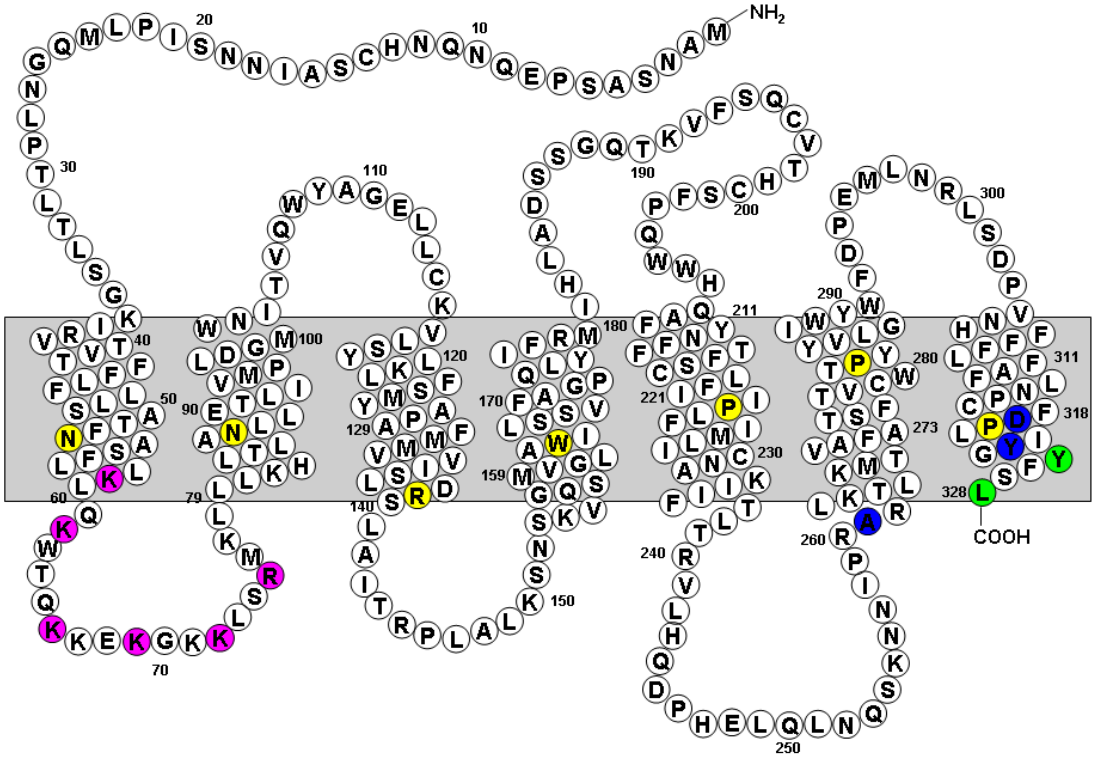


Figure 4.7. Secondary structure representation of the human GnRH receptor. This figure is a two-dimensional representation of the human GnRH receptor revealing the 7 transmembrane-spanning regions and intra- and extracellular loops. The most highly conserved residues in the rhodopsin family of GPCRs are indicated in yellow. Residues that are mutated and cause $G_{q/11}$ uncoupling (see section 4.4.5) are in blue. Amino acids that constitute the YxxL motif are in green and residues that make up putative src SH3 binding (R/K)xx(K/R) motifs are indicated in pink.

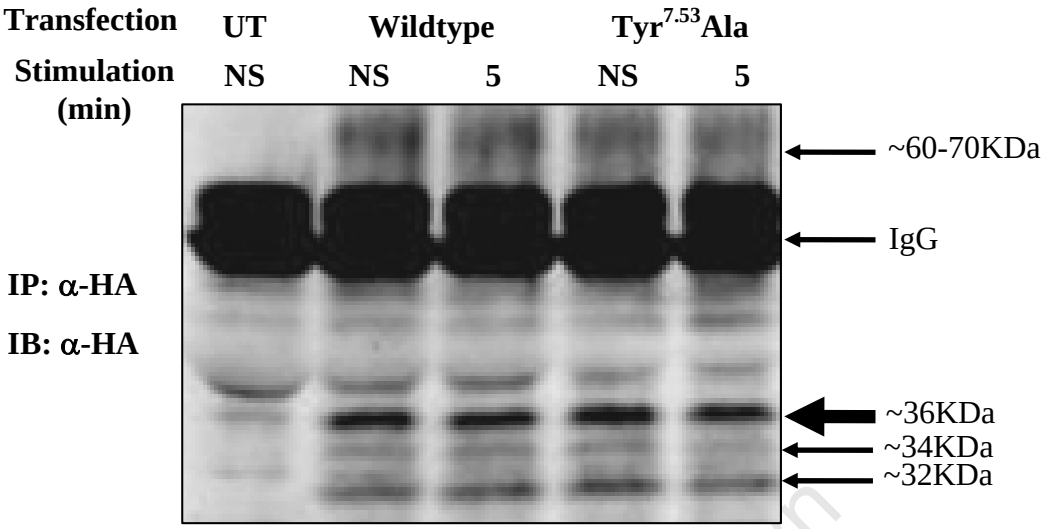


Figure 4.8. Expression of the GnRH receptor in MCF-7 cells. MCF-7 cells were transfected with HA-tagged (at the N-terminus) wildtype and Tyr$^{7.53}$Ala GnRH receptors. Untransfected (UT) cells were used as a negative control. Cells were serum-starved overnight and stimulated with 100nM GnRH I or with vehicle for 5 minutes. GnRH receptors were immunoprecipitated with an anti-HA antibody and immunoblotted with the same antibody. Four GnRH receptor bands are present and are indicated with arrows and approximate sizes in kilodaltons (kDa). The most prominent GnRH receptor band is indicated with the largest arrow. The heavy chain IgG band from the antibody used to immunoprecipitate the receptor is also indicated. This figure is representative of three independent experiments.

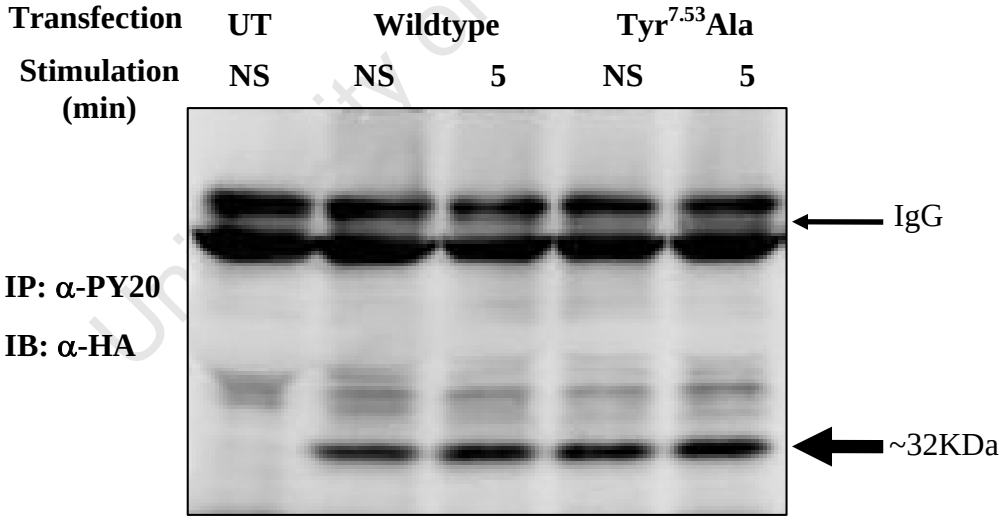


Figure 4.9. Tyrosine phosphorylation of the wildtype and Tyr$^{7.53}$Ala GnRH receptors. MCF-7 cells were transfected with the HA-tagged wildtype and Tyr$^{7.53}$Ala GnRH receptors. UT cells were used as a negative control. Cells were serum-starved overnight and stimulated with 100nM GnRH I or with vehicle for 5 minutes. Tyrosine phosphorylated proteins were immunoprecipitated with the PY20 antibody and GnRH receptors detected with an anti-HA antibody. The specific GnRH receptor band detected is indicated with a large arrow and its approximate size in KDa. This figure is representative of three independent experiments.

4.4.7 Determination of src binding to the GnRH receptor

Assuming that the pYxxL motif represents the mechanism whereby SHP-2 interacts with and is activated by the GnRH receptor, it was necessary to address how SHP-2 undergoes Tyr phosphorylation following its recruitment to the receptor in response to agonist. Previous experimental evidence led to the investigation of the role of the cytoplasmic tyrosine kinase, src, in this phosphorylation event. Firstly, src has been shown to phosphorylate SHP-2 on Tyr^{542} in other cell lines (Li et al., 2006). Furthermore, in other GPCR systems, such as the somatostatin receptors, SHP-2 activation required src activation (Ferjoux et al., 2003; Florio, 2008).

Thus, to determine if src is responsible for the GnRH I-elicited phosphorylation of SHP-2, receptors were stimulated with GnRH I following preincubation in the presence or absence of the src inhibitor, PP2. Compared with control cells, PP2 preincubation significantly ($p<0.01$) decreased basal SHP-2 phosphorylation (Fig.4.10). This suggests that src is primarily responsible for SHP-2 phosphorylation on Tyr^{542} in MCF-7 cells. Furthermore, PP2 prevented GnRH I-elicited SHP-2 phosphorylation (Fig.4.10), suggesting that src is required for GnRH receptor-mediated phosphorylation of SHP-2. These data thus suggest that src phosphorylates SHP-2 on Tyr^{542} in response to GnRH I.

As src is suggested to phosphorylate SHP-2 and GnRH I-elicited SHP-2 phosphorylation occurs independently of G proteins, it was reasoned that src activity may also be regulated by direct interaction with the GnRH receptor. Indeed, activation of src by direct engagement with other GPCRs has been shown previously (Cao et al., 2000; Fan et al., 2001; Sun et al., 2007a). Furthermore, multimeric src-SHP complexes have been shown to occur at the somatostatin GPCRs (Arena et al., 2007; Ferjoux et al., 2003). Thus, the ability of src to form a direct binding complex with the GnRH receptor was assessed. Src was immunoprecipitated from MCF-7 cells transiently transfected with the HA-tagged wildtype GnRH receptor, and co-immunoprecipitation of the GnRH receptor determined. The results show that src pulled down four GnRH receptor bands (Fig.4.11) corresponding to all the isoforms of the GnRH receptor expressed in these cells (see Fig.4.8). These bands were not observed in the untransfected cells showing that the bands observed are specific for

the GnRH receptor. These results show that src is able to form a binding complex at the GnRH receptor and that, like tyrosine phosphorylation of the GnRH receptor, this association is constitutive and does not increase considerably upon GnRH I stimulation (Fig.4.11). Furthermore, the results showed that src is able to associate with all four GnRH receptor isoforms. Interestingly, compared with detection of GnRH receptor expression where the most intense band was ~36KDa (Fig.4.8), the most prominent GnRH receptor associated with src corresponds to ~32KDa, the isoform of the GnRH receptor which is tyrosine phosphorylated (see Fig.4.9). This result suggests that, while src association with the receptor does not require Tyr phosphorylation, src is preferentially associated with the Tyr phosphorylated isoform of the GnRH receptor. This may be because src has a higher affinity for the phosphorylated GnRH receptor isoform and/or because src is responsible for GnRH receptor phosphorylation.

There are various mechanisms that enable GPCRs to form a direct complex with src, including engagement of the SH2 or SH3 domains of the kinase or by formation of a GPCR-β-arrestin-src binding complex (Cao et al., 2000; Fan et al., 2001; Luttrell et al., 1999). The above result shows that the pYxxL motif is not critical for src association as src associates with all four GnRH receptor isoforms. This suggests that src does not associate with the GnRH receptor via its SH2 domain (Fig.4.11). Additionally, robust GnRH receptor-mediated SHP-2 phosphorylation was detected in COS-7 cells which have low endogeneous β-arrestins (Fig.S6) and previous evidence in the literature, as indicated by internalisation and signalling studies, suggest that the human GnRH receptor does not interact with β-arrestins (Caunt et al., 2006; McArdle et al., 1999). This suggests that it is unlikely that src utilises a β-arrestin scaffold for interaction with the GnRH receptor. Thus, together, these results suggest that src associates with the GnRH receptor via its SH3 domain. Analysis of the GnRH receptor amino acid sequence for putative SH3 binding motifs was undertaken. Sequence analysis of the GnRH receptor revealed the presence of three (R/K)xx(K/R) motifs in ICL1 (Fig.4.7), which represent putative SH3 binding domains (Li, 2005; Seet et al., 2007). Thus it is proposed that the GnRH receptor engages with the src SH3 domain via these highly basic consensus motifs.

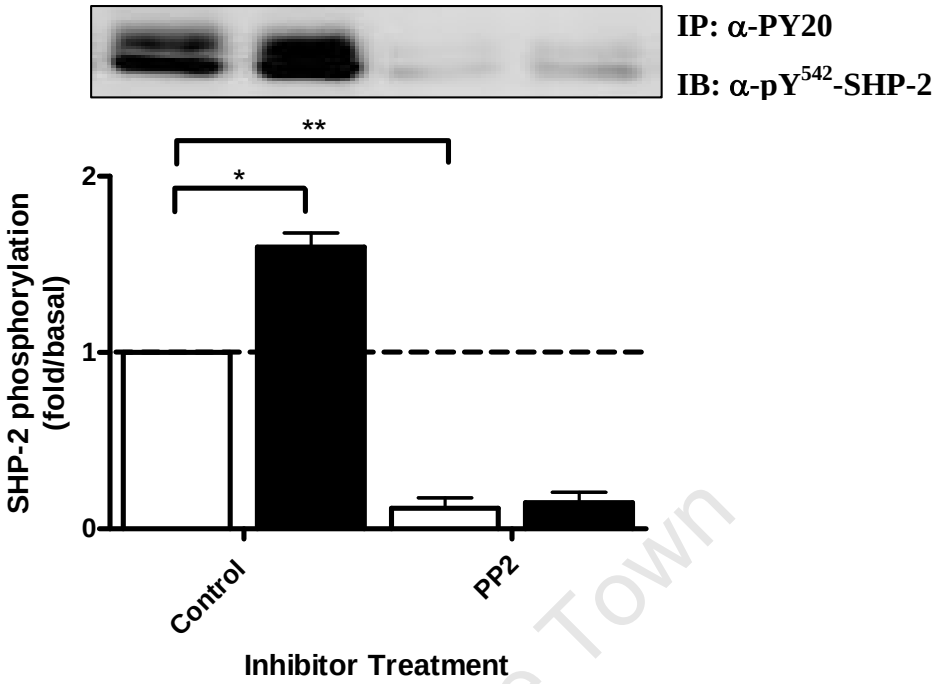


Figure 4.10. The effect of the src inhibitor, PP2, on GnRH I-elicited SHP-2 phosphorylation. MCF-7 cells were transiently transfected with the GnRH receptor and serum-starved as described previously. Prior to stimulations, cells were pretreated with 5μM PP2 or DMSO (for control cells) for 30 minutes. Cells were stimulated with GnRH I (filled bars) or vehicle (NS controls) (open bars) for 5 minutes. Data represent the mean ± S.E. of three independent experiments. *, $p<0.05$, **, $p<0.01$, bracketed bars significantly different; Students *t* test.

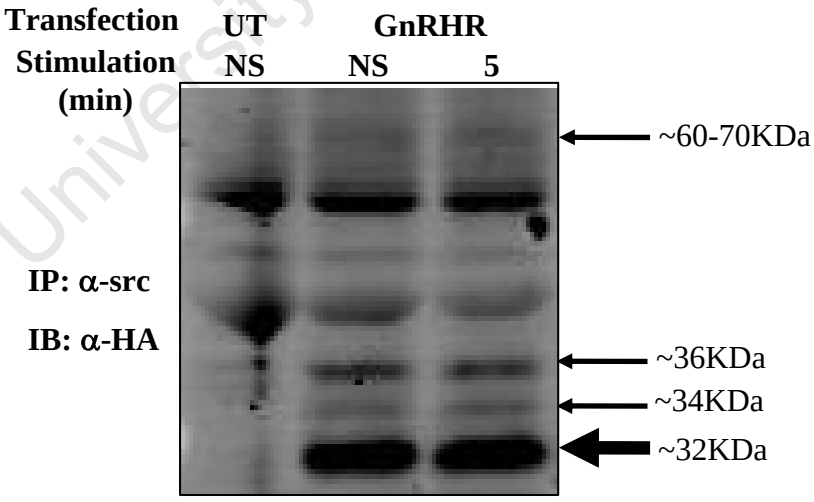


Figure 4.11. Determination of src association with the GnRH receptor. Serum-starved transiently transfected MCF-7 cells were stimulated with 100nM GnRH I or vehicle (NS control) for 5 minutes. Src was immunoprecipitated with an antibody which recognises its N-terminus and associated GnRH receptors were detected with an anti-HA antibody. Four GnRH receptor bands are present and are indicated with arrows and the approximate sizes in kDa. The most prominent GnRH receptor band is indicated with a larger arrow. Untransfected (UT) MCF-7 cells served as a negative control. This figure is representative of three independent experiments.

4.4.8 Effects of GnRH I-elicited SHP-2 activation on downstream ERK activation

In order to establish the downstream signalling targets of SHP-2 activation in response to GnRH I and thereby begin to determine the functional and physiological significance of this novel pathway, known targets/pathways of SHP-2 that overlap with established GnRH receptor signalling pathways were examined. Both the GnRH receptor and SHP-2 are well-established activators of the MAPK pathway and ERK signalling (Caunt et al., 2006; Chong and Maiese, 2007). The activation of ERK plays a role in a number of important cellular processes, such as cell growth and proliferation (Kimura et al., 1999; Meloche and Pouyssegur, 2007) and transcription (Caunt et al., 2006; Maudsley et al., 2007). Thus the role of GnRH I-elicited SHP-2 activation on downstream ERK signalling was investigated.

MCF-7 cells were transfected with the GnRH receptor and a vector control or SHP-2 dominant-negative construct, SHP-2(c/s), which has a non-functional phosphatase domain (Kolli et al., 2004), and the ability of the GnRH receptor to elicit ERK activation was evaluated (Fig.4.12). In control cells, GnRH I induced robust statistically significant ERK activation that was maximal at 10 minutes ($p<0.01$) and decreased at the subsequent 30 and 60 minute time points, but was still elevated above basal levels at 60 minutes of GnRH I stimulation. The kinetics of this ERK response is comparable with that observed in several other cell lines (Benard et al., 2001; Caunt et al., 2006). In cells transfected with the dominant-negative SHP-2(c/s) construct, basal ERK phosphorylation was reduced (Fig.4.12) consistent with the established role of SHP-2 in ERK signalling (Chong and Maiese, 2007). Nevertheless, the increase in GnRH I-elicited ERK phosphorylation in the presence of the SHP-2(c/s) construct was robust and exhibited similar kinetics to that observed with the control cells (Fig.4.12). This result shows that SHP-2 does not make a major contribution to the robust ERK response induced by the GnRH receptor and is consistent with previous reports showing that the GnRH receptor-mediated ERK activation occurs mainly via a PKC-dependent mechanism in several cell lines (Benard et al., 2001; Caunt et al., 2006; Kraus et al., 2001; Naor et al., 2000). However, interestingly, while the difference is small, the increase in GnRH I-elicited ERK activation at the 30 and 60 minute time points were consistently lower in the SHP(c/s)-expressing cells, compared with controls in each experiment, resulting in a

statistically significant difference ($p<0.01$ and $p<0.05$ for 30 and 60 minute time points respectively). This result shows that SHP-2 activation contributes to ERK activation by the GnRH receptor at 30 and 60 minutes and thus SHP-2 facilitates a more sustained ERK response by the GnRH receptor.

4.4.9 Effects of GnRH I-elicited SHP-2 activation on downstream Akt phosphorylation

Another signalling pathway that is regulated by both the GnRH receptor and SHP-2 is the Akt signalling pathway (Chong and Maiese, 2007; Kraus et al., 2004; Rose et al., 2004). Akt signalling is important for several fundamental cellular functions, including transcription, proliferation, growth and survival (Hawkins et al., 2006; Osaki et al., 2004). Previous reports suggest that the GnRH receptor inhibits Akt phosphorylation on Ser^{473}, and thus activation of Akt, in several cell lines (Kraus et al., 2004; Rose et al., 2004). In contrast, SHP-2 has been shown to activate or inhibit Akt signalling under different cellular conditions (Chong and Maiese, 2007). Thus, the effect of GnRH I-elicited SHP-2 activation on Akt signalling was examined.

Activation of Akt by phosphorylation on Ser^{473} in response to GnRH I was assessed in the presence of expression of a vector control or the dominant negative SHP-2(c/s) construct. In the control cells, the GnRH receptor induced a small transient increase in Akt phosphorylation on Ser^{473} that was maximal at 10 minutes and decreased at the subsequent 30 and 60 minute time points (Fig.4.13). However, this response was not statistically significant. In contrast, in the SHP-2(c/s)-expressing cells, the phosphorylation of Akt on Ser^{473} was enhanced and was statistically significant at 10 ($p<0.01$) and 30 ($p<0.05$) minutes of GnRH I stimulation (Fig.4.13). This result suggests that activation of SHP-2 by the GnRH receptor may contribute to the inhibition of Akt activation, which involves its phosphorylation on Ser^{473}.

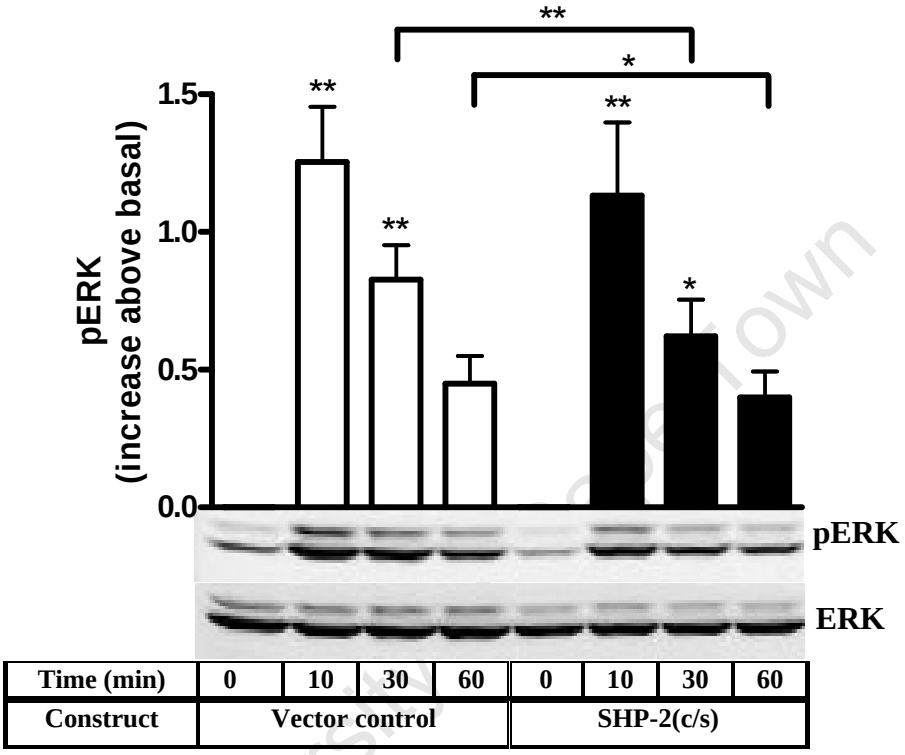


Figure 4.12. The effects of a SHP-2 dominant negative construct, SHP-2(c/s), on GnRH I-elicited ERK activation. MCF-7 cells were transfected with the GnRH receptor and a vector control (for control cells) or SHP-2(c/s) at a 1:1 ratio. Cells were serum-starved overnight and then stimulated with 100nM GnRH I for the indicated time points. Bars represent phosphorylated ERK (pERK) normalised for total ERK (which serves as a loading control) and following removal of basal pERK values. Points are the mean ± S.E. of at three independent experiments. *, $p<0.05$, **, $p<0.01$, significantly different from NS controls (one-way ANOVA with Dunnett's multiple comparison test) or comparison of bracketed bars (Students *t* test).

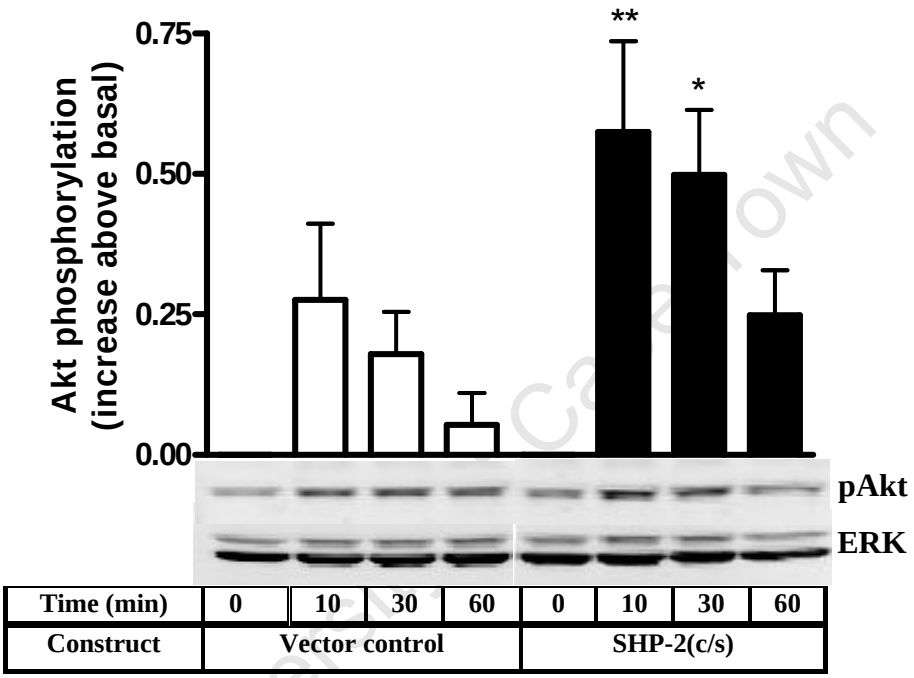


Figure 4.13. The effect of a SHP-2 dominant negative construct, SHP-2(c/s) on Ser^{473} phosphorylation of Akt in response to GnRH I. MCF-7 cells were transfected with the GnRH receptor and a vector control or SHP-2(c/s) at a 1:1 ratio. Cells were serum-starved and stimulated with 100nM GnRH I for the indicated times. Akt phosphorylation on Ser^{473} was detected with a phosphospecific antibody. Bars represent phosphorylated Akt (pAkt) normalised for total ERK (which serves as a loading control) and following removal of basal pAkt values. Points are the mean ± S.E. of at three independent experiments. *, $p<0.05$, **, $p<0.01$, significantly different from NS controls; one-way ANOVA with Dunnett's multiple comparison test.

4.5 Discussion

The GnRH receptor exhibits anti-proliferative and proapoptopic effects in reproductive cancer cells (Grundker and Emons, 2003; Grundker et al., 2001; Kraus et al., 2004; Maiti et al., 2005; Maudsley et al., 2004). Activation of the GnRH receptor stimulates PTP activity and has been shown to interfere with the proliferative activity of several RTKs (Grundker et al., 2001; Imai et al., 1996a; Imai et al., 1996b; Marelli et al., 1999; Moretti et al., 1996). This effect is believed to facilitate the anti-proliferative effects mediated by this receptor. Interestingly, anti-proliferative signalling mediated by the GnRH receptor doesn't correlate with activation of the classical $G_{q/11}$ signalling pathway (Grundker and Emons, 2003; Millar et al., 2008), suggesting that another pathway is responsible for these effects. In light of emerging evidence suggesting that GPCRs are able to make direct interactions with the PTPs, SHP-1 and SHP-2 (Duchene et al., 2002; Ferjoux et al., 2003; Lopez et al., 1997; Vatinel et al., 2006), the present study aimed to investigate whether the GnRH receptor was able to mediate activation of these PTPs.

4.5.1 GnRH I elicits SHP-2 phosphorylation at the GnRH receptor in a time- and dose-dependent manner

To ascertain if the GnRH receptor was able to activate the PTPs, SHP-1 and SHP-2, tyrosine phosphorylation of their C-terminal tails on Tyr^{536} and Tyr^{542} respectively, in response to GnRH I, was assessed. Determination of SHP-1 activation, by evaluating its phosphorylation on Tyr^{536}, was unsuccessful. Neither basal nor GnRH I-elicited SHP-1 phosphorylation was detected (Fig.S3), despite reports that SHP-1 is expressed in these cells (Thangaraju et al., 1999). It may be that the antibody used has low affinity or that phosphorylation of this residue is a rare event, as SHPs can be activated by other mechanisms in addition to phosphorylation on the C-terminal tail (Neel et al., 2003). Thus this result does not preclude the possibility that the GnRH receptor can activate SHP-1.

In contrast, the results show that GnRH I elicited a robust and specific phosphorylation of SHP-2 on Tyr^{542} in a time- and dose-dependent manner in MCF-7 cells (Fig.4.1 and Fig.4.2). Analysis of the kinetics of SHP-2 phosphorylation shows that the response is rapid suggesting that this is a proximal signalling event.

Furthermore, the protracted activation over 60 minutes indicates that this signalling pathway is not readily downregulated. GnRH I had a high potency for the activation of SHP-2 with an EC_{50} value of ~1nM. Together, the robust, rapid and high potency of the GnRH I-elicited SHP-2 response suggest that this pathway is likely to represent a physiologically relevant signalling event.

4.5.2 GnRH I-elicited SHP-2 phosphorylation occurs independently of EGFR transactivation and is only partially dependent on G protein signalling

To investigate the mechanism whereby the GnRH receptor mediates SHP-2 activation, several signalling pathways were examined. First, considering that the EGF receptor is a well-established activator of SHP-2, the possibility that the GnRH receptor activates SHP-2 by transactivation of the EGF receptor, was investigated. The results show that the GnRH receptor was able to induce robust SHP-2 activation in the presence of an EGFR inhibitor (Fig.4.3). Thus, it was concluded that the ability of the GnRH receptor to activate SHP-2 in the MCF-7 cells is not via transactivation of the EGFR.

Next, the role of G protein signalling in GnRH receptor-mediated SHP-2 activation was explored. Several experimental results show that SHP-2 activation by the GnRH receptor can be mediated independently of G proteins. Firstly, inhibitors of the Gα proteins, $G_{q/11}$ and G_i, did not completely prevent SHP-2 activation in response to GnRH I (Fig.4.4). Furthermore, overexpression of the Gβγ subunits, which effectively switch off the activity of the Gα subunits (Cabrera-Vera et al., 2003), did not completely prevent SHP-2 activation (Fig.4.4) supporting the proposal that this pathway can be activated independently of G proteins. Additional evidence is the ability of the $G_{q/11}$-uncoupled $Tyr^{7.53}Ala$ and $Asp^{7.49}Ala$ mutant GnRH receptors to induce robust SHP-2 phosphorylation comparable with the wildtype receptor (Fig.4.6). Furthermore, a classical GnRH receptor antagonist at the $G_{q/11}$ signalling pathway, Ant135-18, was able to induce SHP-2 phosphorylation at the GnRH receptor (Fig.4.5). Taken together, these results show that the GnRH receptor can mediate SHP-2 activation independently of G protein coupling.

An important observation is that the $G_{q/11}$ inhibitor and the Gβγ subunits, while unable to prevent SHP-2 phosphorylation, nevertheless reduced the SHP-2 response and the extent of the inhibition was comparable (Fig.4.4). This suggests that there is a partial dependence on $G_{q/11}$ for SHP-2 activation by the GnRH receptor. Nevertheless, a second explanation exists. The $G_{q/11}$ inhibitor is proposed to inhibit $G_{q/11}$ signalling by preventing GDP/GTP exchange, but doesn't affect the ability of $G_{q/11}$ to interact with the receptor (Takasaki et al., 2004). Thus the inhibitor may inhibit SHP-2 activation by preventing G protein dissociation from the receptor, thereby precluding access of SHP-2 to the receptor. The Gβγ subunits, which promote association of the Gα subunits with the receptor (Cabrera-Vera et al., 2003), may thus inhibit SHP-2 activation in a similar manner. This proposal is supported by the observation that the steric constraint imposed by the $Ala^{6.29}$Lys mutation inhibited both $G_{q/11}$ and SHP-2 activation, suggesting that $G_{q/11}$ and SHP-2 may interact with an overlapping region/domain of the GnRH receptor and thus that their binding is mutually exclusive.

Classically, GPCRs, as their name suggests, are thought to require activation of G proteins in order to activate intracellular signalling cascades. However, emerging evidence in several GPCRs has revealed that this family of receptors may also utilise G protein-independent signalling mechanisms, particularly in the activation of phosphotyrosine signalling pathways (Sun et al., 2007a; Sun et al., 2007b). Furthermore, this mechanism of GPCR signalling is gaining recognition as a physiologically relevant pathway *in vivo* (Zhai et al., 2005). My results show that the GnRH receptor is able to activate SHP-2 in a G protein-independent manner, thus presenting another example of a GPCR which can signal via non-classical signalling mechanisms and extending the number of GPCRs that exhibit G protein-independent signalling. In the context of other GPCRs which signal by G protein-independent mechanisms, these results provide further support for the proposal that G protein-independent signalling may represent a more broadly utilised mechanism of GPCR signalling.

4.5.3 The GnRH receptor conformations that mediate SHP-2 and $G_{q/11}$ activation are distinct

A further implication inherent in the above results is that the GnRH receptor is able to adopt multiple active conformations and that these conformations have distinct abilities to activate the $G_{q/11}$ and SHP-2 signalling pathways. For example, the ability of Ant135-18 to activate SHP-2, but not $G_{q/11}$ signalling, suggests that this ligand stabilises a receptor conformation that can facilitate SHP-2, but not $G_{q/11}$, activation. Furthermore, alanine mutagenesis of $Tyr^{7.53}$ and $Asp^{7.49}$ revealed that these residues are required to make intramolecular interactions necessary for a receptor conformation that enables $G_{q/11}$ signalling (Arora et al., 1996; Flanagan et al., 1999; Lu et al., 2005), but the results presented here show that they are not important for GnRH receptor-mediated SHP-2 activation (Fig.4.6). Thus these data also support the proposal that the GnRH receptor can adopt distinct active conformations with different signalling capabilities. The ability of the GnRH receptor to adopt multiple receptor conformations with distinct signalling capabilities has important therapeutic implications as it presents the exciting possibility that drugs can be developed to specifically target desired signalling pathways, without activating signalling which causes adverse effects.

4.5.4 The GnRH receptor exhibits a constitutively tyrosine-phosphorylated canonical SH2-binding pYxxL motif

The ability of the GnRH receptor to activate SHP-2 independently of G proteins suggests that SHP-2 is activated by direct interaction with the GnRH receptor. Indeed, SHP-2 has been shown to interact directly with SH2-binding motifs in a few other GPCRs, including the bradykinin, somatostatin and chemokine CCK2 receptors (Duchene et al., 2002; Ferjoux et al., 2003; Vatinel et al., 2006). Analysis of the GnRH receptor amino acid sequence identified a putative SH2-binding YxxL motif at the C-terminal end of TM7 (Fig.4.7) and further examination of tyrosine phosphorylation of the wildtype and $Tyr^{7.53}$Ala GnRH receptors revealed that $Tyr^{7.55}$ within this YxxL motif is phosphorylated indicating that this motif has the hallmarks of a canonical SH2-binding domain (Fig.4.9). It is thus proposed that SHP-2 interacts with and is activated by the GnRH receptor via this pYxxL motif in TM7 of the GnRH receptor.

There are several other important observations regarding the tyrosine phosphorylation of the GnRH receptor. Firstly, tyrosine phosphorylation of the GnRH receptor was constitutive and, while four GnRH receptor isoforms were expressed in the MCF-7 cells (Fig.4.8), only one of these isoforms was tyrosine phosphorylated (Fig.4.9). This suggests that, in the basal state, GnRH receptors exist as a heterogeneous population of differentially Tyr phosphorylated states. An important consequence of the identification of a subpopulation of GnRH receptors that exhibit constitutive tyrosine phosphorylation is the possibility that the distribution of GnRH receptors in the phosphorylated state may vary in different tissues. Expression of the GnRH receptor in cancerous tissues, where the activity of tyrosine kinases, such as src, is elevated, may contribute to a higher proportion of phosphorylated receptors and thus facilitate tissue-specific signalling of the receptor (Maudsley et al., 2005; Nelson and Challiss, 2007). Indeed, the M_3-muscarinic receptor is differentially phosphorylated by casein kinase 2 (CK2) in different cell types (Torrecilla et al., 2007) and CK2 phosphorylation of this receptor inhibits its activation of Jun-kinase signalling, but doesn't affect ERK activation or receptor internalisation (Torrecilla et al., 2007). Thus, the tissue-specific expression, localisation and regulation of kinases can differentially affect the signalling outcome of receptor activation in different cell types.

The effects of tyrosine phosphorylation on GnRH receptor signalling have not been explored. However, it has been reported that tyrosine phosphorylation of the GnRH receptor may regulate receptor conformation (Liebow et al., 1991). Specifically, it has been shown that tyrosine phosphorylation of the human GnRH receptor alters the binding of the GnRH agonist, [D-Trp^6]GnRH (Liebow et al., 1991). In the presence of EGF and ATP, phosphorylation of the GnRH receptor was observed and correlated with an increase in [D-Trp^6]GnRH binding. Similarly, incubation with somatostatin analogue, RC-160, facilitated dephosphorylation of the GnRH receptor and induced a decrease in [D-Trp^6]GnRH binding. However, no change in GnRH receptor affinity was observed and as these experiments were performed on pancreatic cancer cell membranes, altered receptor trafficking or protein synthesis cannot explain this phenomenon (Liebow et al., 1991). The authors propose an

allosteric mechanism, where phosphorylation alters receptor conformation, shifting the receptor conformational equilibrium to favour the high affinity binding state. This suggests that phosphorylation of the GnRH receptor could alter the potency of GnRH receptor signalling by increasing the number of high affinity receptors.

Thus, these results show that the human GnRH receptor exhibits constitutive tyrosine phosphorylation in MCF-7 cells. This introduces a new level of complexity and may contribute to tissue-specific signalling observed at the GnRH receptor. Furthermore, $Tyr^{7.55}$ has been identified as the Tyr residue which is phosphorylated and thus indicates that the pYxxL motif in TM7 of the GnRH receptor represents a canonical SH2-binding domain. This site thus represents the proposed mechanism whereby SHP-2 interacts directly with the GnRH receptor and is activated independently of G proteins.

4.5.5 $Tyr^{7.53}$ of the D/NPxxY motif constitutes a molecular switch that regulates SHP-2 activation

The suggestion that the pYxxL motif is important for SHP-2 activation is consistent with the experimental data observed at the $Tyr^{7.53}$Ala mutant receptor. This receptor exhibited elevated basal SHP-2 phosphorylation levels compared with the wildtype receptor, suggesting that this receptor has constitutive activity at the SHP-2 signalling pathway (Fig.4.6). This further supports the proposal that the GnRH receptor conformations that mediate SHP-2 and $G_{q/11}$ signalling are distinct, as this mutant is not constitutively active at the $G_{q/11}$ signalling pathway. Additionally, this result suggests that $Tyr^{7.53}$ interactions contribute to a molecular switch that constrains a functional domain which facilitates SHP-2 activation. I have proposed that the functional domain that facilitates SHP-2 activation is the pYxxL at the C-terminal end of TM7 of the GnRH receptor. Previous reports suggest that the analogous Tyr participates in a conformational switch that specifically alters the conformation of the functional domain constituting the C-terminal tail in other GPCRs (Kalatskaya et al., 2004; Prioleau et al., 2002; Weinstein, 2005). For example, alanine mutagenesis of the equivalent residue in the B_2 Bradykinin receptor led to constitutive phosphorylation and internalisation of the receptor, but did not induce constitutive $G_{q/11}$ signalling (Kalatskaya et al., 2004). Thus, I propose that, in

a similar way, alanine mutagenesis of $Tyr^{7.53}$ in the GnRH receptor disrupts a molecular switch, which constrains the position of the pYxxL motif at the bottom of TM7, thereby enabling constitutive activation of SHP-2, but not $G_{q/11}$ signalling.

4.5.6 Src forms a direct and constitutive binding complex with the GnRH receptor and is necessary for GnRH I-elicited SHP-2 activation

Tyrosine phosphorylation of the GnRH receptor and SHP-2 in response to GnRH I supported the involvement of a tyrosine kinase in this signalling pathway. Due to the reported regulation of SHP-2 by src (Li et al., 2006), it was considered feasible to investigate the participation of src in this signalling pathway. The results presented here show that the src inhibitor, PP2, decreased both basal SHP-2 phosphorylation and GnRH I-elicited SHP-2 phosphorylation (Fig.4.10). Thus it was concluded that src is responsible for SHP-2 phosphorylation in MCF-7 cells.

The involvement of src in the SHP-2 signalling pathway introduced a further mechanistic challenge regarding how this protein is regulated by the GnRH receptor independently of G proteins. Nevertheless, recent reports suggest that GPCRs are also able to directly interact with src either via a β-arrestin scaffold, or by interaction with the SH2 or SH3 domains of src (Cao et al., 2000; Fan et al., 2001; Luttrell et al., 1999). Indeed, immunoprecipitation experiments revealed that src associated with all four GnRH receptor isoforms expressed in the MCF-7 cells (Fig.4.11). The experimental data excluded the possibility that src associates with the GnRH receptor via its SH2 domain as tyrosine phosphorylation of the GnRH receptor was not an absolute requirement for src association (Fig.4.11). Furthermore, the ability to detect robust SHP-2 phosphorylation in COS-7 cells with low endogeneous β-arrestins suggested that src does not associate with the GnRH receptor via a β-arrestin scaffold. Analysis of the GnRH receptor sequence revealed the presence of three (R/K)xx(K/R) motifs in ICL1 (Fig.4.7) which represent putative SH3 binding domains (Li, 2005; Seet et al., 2007). Thus, it is proposed that src associates via its SH3 domain with (R/K)xx(K/R) motifs in ICL1 of the GnRH receptor.

There are two other important observations regarding src association with the GnRH receptor. Firstly, like tyrosine phosphorylation of the GnRH receptor, the association

of src with the GnRH receptor was constitutive. Secondly, while src bound to all four GnRH receptor isoforms, it was preferentially associated with the phosphorylated receptor state (Fig.4.11). This result shows that phosphorylation of the receptor is not required for src binding, but is nevertheless highly correlated with this event. A proposed mechanism which explains this phenomenon is that src is responsible for the tyrosine phosphorylation of the GnRH receptor. Thus src binding to the GnRH receptor may facilitate its activation thereby enabling phosphorylation of the GnRH receptor.

4.5.7 Proposed model of GnRH receptor-mediated SHP-2 activation

Thus from the above results, I propose the following model for GnRH I-elicited SHP-2 activation at the GnRH receptor (Fig.4.14). In the basal state, src associates with ICL1 of the GnRH receptor via its SH3 domain. This event facilitates activation of src and enables src to phosphorylate $Tyr^{7.55}$ of the YxxL motif in TM7 of the GnRH receptor, creating a docking site for SHP-2. SHP-2 either constitutively associates with this motif or ligand binding and a receptor conformational rearrangement is required in order for SHP-2 to access the site. Upon GnRH I stimulation, the GnRH receptor undergoes a conformational change involving disruption of the constraining interactions of $Tyr^{7.53}$ of the D/NPxxY motif. This conformational change either allows SHP-2 access to the pYxxL motif or facilitates a change in the relative conformation of src and SHP, thus enabling src phosphorylation of Tyr^{542} on SHP-2 and activation of the phosphatase.

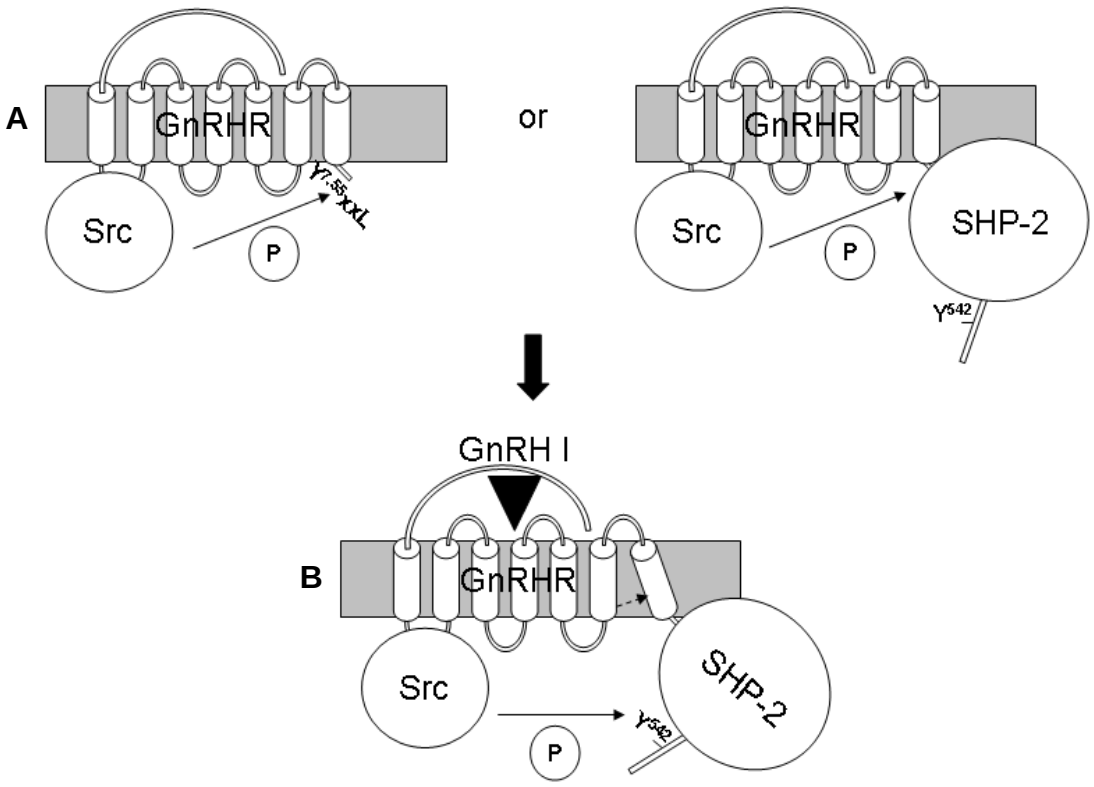


Figure 4.14. Schematic representation of the proposed mechanism of SHP-2 activation by the GnRH receptor in response to GnRH I. In the basal state (A), src binds to ICL1 of the GnRH receptor via its SH3 domain. This facilitates activation of the kinase and enables src phosphorylation of $Tyr^{7.55}$ of the YxxL motif in TM7 of the GnRH receptor, creating a docking site for SHP-2. SHP-2 either binds to this motif in the basal state or ligand-induced receptor conformational changes are required to allow SHP-2 access to this site. Following GnRH I binding (B), the GnRH receptor undergoes a conformational change involving disruption of the constraining interactions made by $Tyr^{7.53}$ of the D/NPxxY motif. This either facilitates SHP-2 binding or changes the relative position of SHP-2 and src, thereby enabling src phosphorylation of SHP-2 on Tyr^{542} and activation of the phosphatase.

This unusual scheme of GnRH receptor signalling is more reminiscent of cytokine activation of the JAK/STAT pathway than the classical GPCR G protein signalling (Haan et al., 2006). However, the number of GPCRs for which direct interactions with src and/or SHP-2 have been demonstrated is increasing, suggesting that this non-classical GPCR signalling may represent a more broadly utilised signalling mechanism (Cao et al., 2000; Duchene et al., 2002; Fan et al., 2001; Ferjoux et al., 2003; Marrero et al., 1998; Olszewska-Pazdrak et al., 2004; Sun et al., 2007a; Sun et al., 2007b). A key question is how the "switch" from G protein-dependent signalling to this G protein-independent mechanism of signalling occurs. It was previously proposed that the G protein-independent activation of src by the β_2-adrenergic receptor involved a dose-dependent switch, where low agonist concentrations activated G proteins, while high agonist concentrations activated the G protein-independent signalling mode (Sun et al., 2007a). However, the low EC_{50} value of ~1nM for GnRH I-elicited SHP-2 activation suggests that this explanation is not sufficient in the GnRH receptor signalling context. I propose a second possible explanation. Specifically, that tyrosine phosphorylation of the GnRH receptor mediates the switch in coupling. This suggestion is supported by the previous observation, discussed above, that phosphorylation of the GnRH receptor alters the number of high affinity GnRH receptors (Liebow et al., 1991), which would thus increase the potency of GnRH receptor signalling. Indeed, PKA phosphorylation of the β_2-adrenergic has been shown to mediate a switch in G protein signalling, from G_s to G_i activation (Daaka et al., 1997). Furthermore, phosphorylation of GPCRs by GRKs mediates a switch from G protein to β-arrestin-mediated signalling (Lefkowitz et al., 2006). Thus the localisation, expression and activity of src and thus phosphorylation of the GnRH receptor in different tissues may dictate the potency of the GnRH receptor G protein-independent signalling to SHP-2.

4.5.8 SHP-2 contributes to GnRH receptor regulation of ERK and Akt signalling

In order to determine the effects of GnRH receptor-mediated SHP-2 activation on downstream signalling, the effects of a SHP-2 dominant negative construct, SHP-2(c/s) on GnRH I-elicited ERK and Akt signalling was assessed. The results show that, in the presence of the SHP-2 dominant negative construct, the ERK response mediated by the GnRH receptor was significantly lower at 30 and 60 minutes of

GnRH I stimulation (Fig.4.12). This result suggests that activation of SHP-2 by the GnRH receptor may increase the magnitude and contribute to a more sustained ERK response. The definitive relevance of this to GnRH receptor signalling requires further investigation, but, in the context of previously performed experimental data, it is possible to speculate that this pathway may contribute to the anti-proliferative signalling mediated by the GnRH receptor. Firstly, ERK activation has been implicated previously in the anti-proliferative signalling of the GnRH receptor (Kimura et al., 1999; Kraus et al., 2006), as well as other GPCRs (Florio et al., 2000; Lahlou et al., 2003). The magnitude and duration of the ERK response, specifically a robust and prolonged activation, facilitates cell cycle arrest by upregulation of cyclin-dependent kinase (Cdk) inhibitors (Meloche and Pouyssegur, 2007). Thus SHP-2 activation may facilitate the anti-proliferative effects of the GnRH receptor by enhancing the magnitude and duration of the ERK response.

Next, the effect of the SHP-2(c/s) on GnRH receptor regulation of Akt activation was assessed. In control cells, Akt activation, as measured by the increase in its phosphorylation on Ser^{473}, was elicited in response to GnRH I, but the response was small and not statistically significant. This small signalling response may be mediated by GnRH receptor transactivation of the EGFR. Interestingly, in the presence of the SHP-2 dominant negative construct, GnRH I-elicited Akt activation was enhanced and the response became statistically significant. This result suggests that SHP-2 may be involved in the inhibition of Akt activation. The Akt signalling pathway plays a central role in the regulation of cell-cycle progression and survival (Hawkins et al., 2006; Osaki et al., 2004). Furthermore, GnRH receptor inhibition of Akt activation has been proposed previously to enable the anti-proliferative effects of the GnRH receptor (Kraus et al., 2004; Rose et al., 2004). Thus, SHP-2 activation by the GnRH receptor contributes to the inhibition of Akt activation and may therefore facilitate the anti-proliferative effects of the GnRH receptor. Thus, together, GnRH receptor-mediated SHP-2 activation, which the results show enables a more robust and prolonged ERK response and facilitates inhibition of Akt signalling, may contribute to the anti-proliferative effects mediated by the GnRH receptor. This is consistent with the involvement of SHP activation in the regulation of cell proliferation by other GPCRs, including the B_2 bradykinin (Duchene et al., 2002),

sst1 and sst2 somatostatin (Ferjoux et al., 2003; Florio, 2008; Florio et al., 2000; Lopez et al., 1997) and CCK2 chemokine receptors (Vatinel et al., 2006).

The above results show that GnRH I-elicited GnRH receptor activation induced both activation and inhibition of Akt signalling, as inferred from Akt phosphorylation on Ser^{473}. This suggests that there are two pathways emanating from the GnRH receptor, a proliferative signalling pathway and an anti-proliferative pathway. Interestingly, while, as discussed, the GnRH receptor exerts anti-proliferative effects in certain cancer cell lines, proliferative effects on other cell lines have been observed (Enomoto et al., 2004). A similar phenomenon has been observed at the B_2 bradykinin receptor, where activation of the receptor results in proliferative or anti-proliferative signalling depending on the experimental conditions (Duchene et al., 2002). In the bradykinin receptor, it has been proposed that the proliferative effects of the receptor are mediated by activation of the $G_{q/11}$ protein, while the anti-proliferative effects are mediated by SHP-2 activation (Duchene et al., 2002). As my results suggest that SHP-2 activation is involved in anti-proliferative signalling of the GnRH receptor, it is tempting to speculate that this $G_{q/11}$-coupled receptor is subject to a similar signalling scheme.

One set of data that is difficult to reconcile with the proposed anti-proliferative effects of SHP-2 activation by the GnRH receptor, is the inability to detect SHP-2 activation in response to Ant135-25 and Cetorelix, which have been shown to mediate anti-proliferative signalling in certain cancer cell lines (Grundker and Emons, 2003; Maudsley et al., 2004). However, the effects of these ligands may be cell-context dependent as other researchers have reported an inability to detect cell death in response to Cetorelix in prostate cancer cells (Maiti et al., 2005). Thus this result does not preclude the possibility that SHP-2 activation by the GnRH receptor facilitates anti-proliferative signalling.

4.5.9 Summary

In summary, the results presented in this chapter show that the GnRH receptor is able to mediate robust time- and dose-dependent activation of SHP-2 in response to GnRH I. The activation of SHP-2 is G protein-independent, but requires src activity

and represents a novel mechanism of GnRH receptor signalling. This signalling pathway contributes to GnRH receptor-mediated ERK and Akt regulation and may thus be involved in the anti-proliferative effects of the GnRH receptor. Of particular interest, SHP-2 activation is mediated by a subset of receptor conformations that are distinct from those that mediate $G_{q/11}$ signalling. Understanding the molecular nature of ligand-receptor interactions and receptor activation relevant to these signalling pathways will create an opportunity for the tailored development of drugs targeted at highly specific GnRH receptor-mediated pathways.

4.6 Supplementary figures

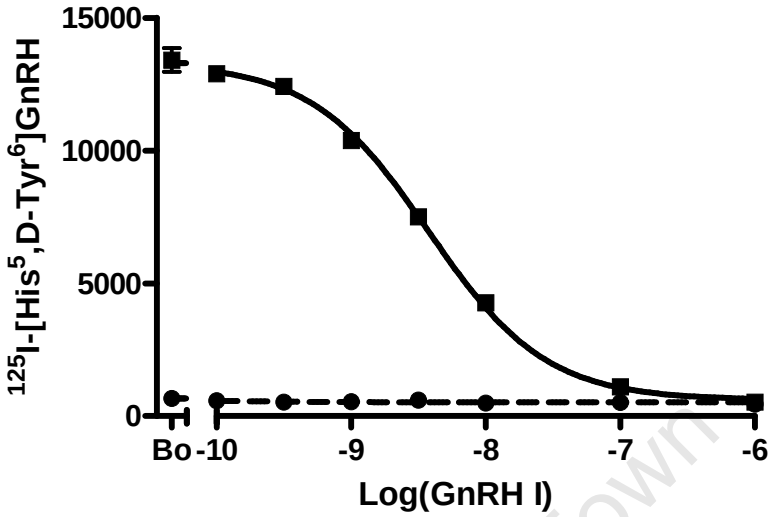


Figure S1. MCF-7 cells exhibit efficient transfection following electroporation. MCF-7 cells were electroporated with 15μg of the wildtype GnRH receptor (■) or control (●) DNA and subjected to whole cell radioligand binding assays 48 hours post transfection (see materials and methods). Control-transfected cells did not exhibit specific binding for the GnRH receptor label, ^{125}I-[His^5,D-Tyr^6]GnRH. However, cells transfected with the wildtype GnRH receptor showed high specific binding of the label indicating efficient transfection and expression of the GnRH receptor in the MCF-7 cells. Furthermore, the IC_{50} value for GnRH I at these receptors was 4nM, which is consistent with that observed in other cell lines (see chapter 2).

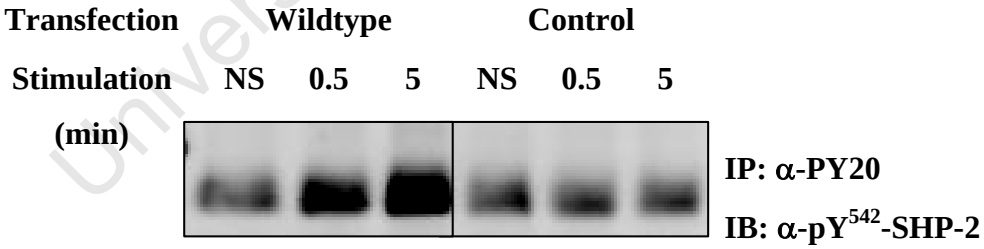


Figure S2. SHP-2 activation in the MCF-7 cells is specifically mediated by the type I GnRH receptor. MCF-7 cells were transfected with control DNA or with the wildtype type I human GnRH receptor. Forty-eight hours following transient transfection and overnight serum starvation, cells were treated with 100nM GnRH I as indicated. Phosphorylated proteins were immunoprecipitated with the PY20 antibody and SHP-2 phoshorylation on Tyr^{542} was detected. Cells transfected with control DNA did not show GnRH I-elicited SHP-2 activation. However, cells transfected with the wildtype receptor showed GnRH I-elicited SHP-2 activation. NS, non-stimulated.

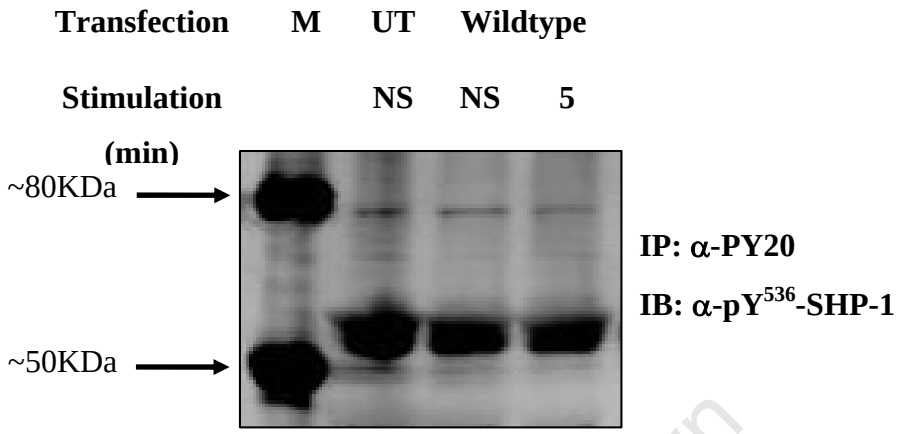


Figure S3. Basal and GnRH I-induced SHP-1 phosphorylation could not be detected in MCF-7 cells. MCF-7 cells were transiently transfected with the wildtype GnRH receptor. Forty-eight hours following transfection and overnight serum starvation, cells were treated with 100nM GnRH I as indicated. Phosphorylated proteins were immunoprecipitated with the PY20 antibody and SHP-1 phoshorylation on Tyr^{536} was detected. The expected molecular weight of SHP-1 is 68KDa. A band of this size could not be detected in these cells using this antibody. M, protein marker; UT, untransfected cells; NS, non-stimulated.

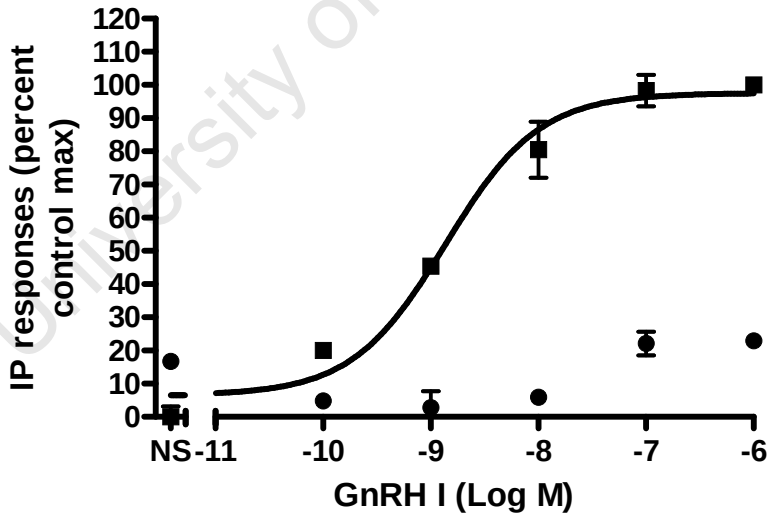


Figure S4. The $G_{q/11}$ inhibitor, YM254890, inhibits GnRH I-elicited inositol phosphate responses at the wildtype GnRH receptor. MCF-7 cells were transiently transfected with the wildtype GnRH receptor. Forty-eight hours following transfection and overnight *myo*-D-[^{3}H]inositol labelling, cells were pre-incubated in the presence of DMSO (■) or 100nM YM254890 (●) for 30 minutes and the GnRH I-elicited inositol phosphate responses were assessed (see materials and methods in chapter 2). Compared with the DMSO control, YM254890 efficiently inhibits GnRH I-elicited inositol phosphate responses over a range of GnRH I concentrations.

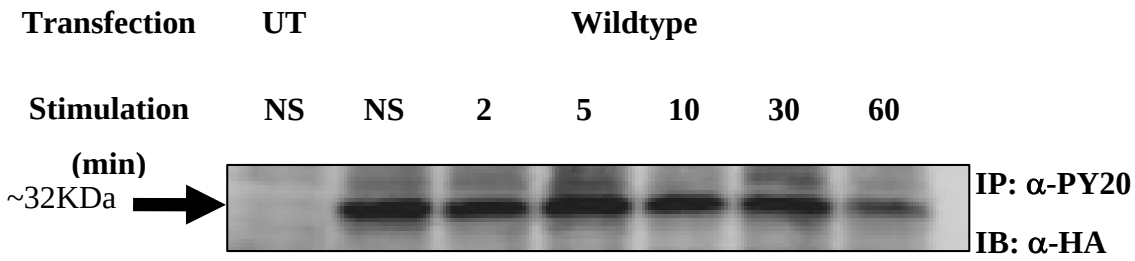


Figure S5. Time-course of GnRH receptor tyrosine phosphorylation in MCF-7 cells. MCF-7 cells were transiently transfected with the HA-tagged wildtype GnRH receptor. Untransfected (UT) cells were used as a negative control. Cells were serum-starved overnight and stimulated with 100nM GnRH I as indicated (forty-eight hours following transfection). Tyrosine phosphorylated proteins were immunoprecipitated with the PY20 antibody and the GnRH receptor was detected with an anti-HA antibody. The specific GnRH receptor band detected is indicated with a large arrow and its approximate size in KDa. The phosphorylation status of the GnRH receptor does not change considerably over a 60 minute time interval.

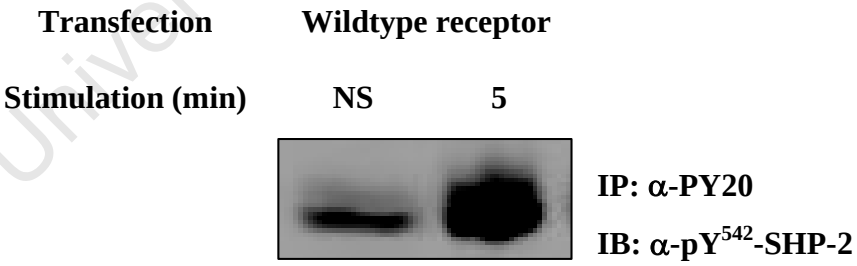


Figure S6. GnRH I elicits robust SHP-2 activation in COS-7 cells. COS-7 cells were transfected with the wildtype GnRH receptor (see materials and methods in chapter 2). Forty-eight hours following transient transfection and overnight serum starvation, cells were treated with 100nM GnRH I as indicated. Phosphorylated proteins were immunoprecipitated with the PY20 antibody and SHP-2 phoshorylation on Tyr^{542} was detected.

5 Chapter 5: Concluding discussion

GnRH receptors are expressed in the pituitary where they regulate reproduction by signalling via $G_{q/11}$ proteins to enable the biosynthesis and release of LH and FSH (Cheng and Leung, 2005; Pawson and McNeilly, 2005). In peripheral reproductive cancer cells, ligand-induced activation of GnRH receptors exerts anti-proliferative effects, which do not correlate with $G_{q/11}$ activation (Grundker and Emons, 2003; Kraus et al., 2006; Limonta et al., 2003). We propose that different ligands stabilise distinct receptor active conformations at the GnRH receptor, which have differential capacity for activation of these downstream signalling pathways and have termed this concept LiSS (Millar et al., 2004; Millar et al., 2008).

Several different types of experimental evidence can be used to support the existence of LiSS at the GnRH receptor. Firstly, evidence supporting the ability of different ligands to stabilise different receptor active conformations are consistent with LiSS at the receptor. Furthermore, showing that different GnRH receptor conformations (induced by ligands or receptor mutations) have differential capacity for activation of downstream signalling pathways can also be used as evidence to support LiSS at the GnRH receptor. In this dissertation, several types of experimental support for LiSS at the GnRH receptor were presented.

In chapter 2, I investigated the ligand-receptor contacts and role of $Tyr^{6.58}$ in facilitating receptor activation in response to GnRH I and GnRH II. Previous reports and our molecular model of GnRH I docked at the GnRH receptor suggested that Tyr^{5} of GnRH I interacts with $Tyr^{6.58}$ of the receptor, but this required experimental validation. GnRH II differs from GnRH I at positions 5, 7 and 8 and has a His in position 5. The importance of $Tyr^{6.58}$ in GnRH II binding also required investigation. My experimental results show that $Tyr^{6.58}$Ala mutation induced large changes in affinity for both GnRHs, but not the Ala^{5}-substituted GnRHs, indicating that $Tyr^{6.58}$ interacts with Tyr^{5} of GnRH I and His^{5} of GnRH II. The $Tyr^{6.58}$Leu and $Tyr^{6.58}$Gln mutant receptors, which mimic the hydrophobic and H-bonding abilities of $Tyr^{6.58}$ respectively, were unable to mediate comparable high affinity binding as observed at the $Tyr^{6.58}$Phe receptor. This emphasises the importance of the aromatic nature of $Tyr^{6.58}$ in the high affinity binding of GnRHs. Thus the results suggest that $Tyr^{6.58}$ interacts with position 5 of the GnRHs by aromatic interactions. In addition to its

importance in ligand binding, mutation of $Tyr^{6.58}$ to Phe and Ala yielded GnRH-induced signalling efficiencies of 82-144% and 23-46% respectively, indicating the importance of the aromatic ring, but not OH group, in receptor activation. The importance of $Tyr^{6.58}$ in receptor activation suggests that this residue participates in intramolecular interactions, in addition to the ligand-receptor contacts, that contribute to the active receptor conformation.

Interestingly, despite the utilisation of $Tyr^{6.58}$ by both GnRH I and GnRH II for ligand binding and receptor activation, the results suggest that the mechanism differs. Firstly, while GnRH I has similar affinity at the $Tyr^{6.58}$Leu and $Tyr^{6.58}$Ala receptors, GnRH II has higher affinity for the $Tyr^{6.58}$Leu receptor, indicating that the Leu side chain at position 6.58 can make compensatory interactions with GnRH II, but not GnRH I. This result is supported by our molecular models of GnRH I and GnRH II docked at the GnRH receptor, which shows that position 5 of the decapeptides interact with $Tyr^{6.58}$ by aromatic interactions with distinct geometries. In the model of GnRH I docked at the receptor, Tyr^{5} makes a T-shaped stacking interaction with $Tyr^{6.58}$, while His^{5} of GnRH II interacts with $Tyr^{6.58}$ by a parallel offset stacking interaction. This arrangement may thus facilitate the ability of the $Tyr^{6.58}$Leu to interact with His^{5} of GnRH II, but not with Tyr^{5} of GnRH I. Additionally, the experimental results show that the $Tyr^{6.58}$Leu receptor cannot mediate receptor activation in response to GnRH I, despite a signalling efficiency of 23% at the $Tyr^{6.58}$Ala receptor. This difference is not due to decreased affinity, as the Leu and Ala receptors have similar affinity for GnRH I. Thus this result suggests that $Tyr^{6.58}$Leu makes intramolecular interactions that prevent formation of the active receptor conformation achieved by the $Tyr^{6.58}$Ala receptor. In contrast, the $Tyr^{6.58}$Leu receptor has higher signalling efficiency (116%) in response to GnRH II than the $Tyr^{6.58}$Ala receptor (46%). This suggests that $Tyr^{6.58}$Leu makes different intramolecular interactions in the GnRH I- compared with the GnRH II-stabilised active receptor conformations. This is also consistent with our molecular models, which show that GnRH I and GnRH II interact with different rotamer conformations of $Tyr^{6.58}$, which thereby enables the formation of the aromatic interactions with distinct geometries discussed above. Thus, together, these data support the proposal that GnRH I and GnRH II stabilise different receptor active conformations and thus

provide evidence that is consistent with LiSS at the GnRH receptor. Furthermore, the results provide valuable verification of the accuracy of our molecular models, which can be used to predict other ligand-receptor and receptor intramolecular interactions that are useful in the understanding of LiSS.

In chapters 3 and 4, I wished to examine the ability of the GnRH receptor to signal to differential downstream signalling pathways that mediate the anti-proliferative signalling effects of the GnRH receptor and are distinct from $G_{q/11}$ activation. This required identification of the most proximal signalling event that mediates the anti-proliferative signalling effects of the GnRH receptor. Previous proposals have suggested that the GnRH receptor mediates anti-proliferative signalling by coupling to the alternative G protein, G_i. Thus, I established a series of [^{35}S]GTPγS binding assays to determine GnRH receptor-G_i coupling. My results show that the GnRH receptor is not able to activate G_i, even in a reconstituted environment of high concentrations of the GnRH receptor and G_i proteins. In contrast, I have identified a novel signalling partner of the GnRH receptor, the SH2 domain-containing phosphatase 2 (SHP-2), which I propose is responsible for the anti-proliferative signalling effects, by forming a direct interaction with the GnRH receptor. The GnRH receptor-induced SHP-2 activation occurred in the presence of a $G_{q/11}$ inhibitor indicating that SHP-2 activation can be activated independently of $G_{q/11}$. Furthermore, the results show that SHP-2 can be activated by the GnRH receptor in response to a classical GnRH receptor antagonist at the $G_{q/11}$ signalling pathway. Thus ligands that are antagonists at the $G_{q/11}$ signalling pathway, can act as agonists at the SHP-2 pathway, suggesting that the two pathways are activated by distinct receptor active conformations and providing further support for LiSS at the GnRH receptor. Additionally, two GnRH mutants, the Tyr$^{7.53}$Ala and the Asp$^{7.49}$Ala receptors, which are uncoupled at the $G_{q/11}$ signalling pathways, were able to mediate activation of SHP-2. These residues are in the TM domains of the receptor and are not proposed to make direct interactions with $G_{q/11}$ or SHP-2, but are rather required to make intramolecular interactions that stabilise the active receptor conformations. Thus the ability of these mutants to mediate differential signalling suggests that these receptors are able to achieve an active receptor conformation that can activate SHP-2, but not $G_{q/11}$. The proposal that these mutants assume different receptor

conformations compared with the wildtype receptor is further supported by the observation that the $Tyr^{7.53}$Ala receptor exhibits constitutive activity at the SHP-2 signalling pathway, but is not constitutively active at the $G_{q/11}$ signalling pathway. Together these results support the proposal that the GnRH receptor is able to adopt multiple distinct receptor active conformations (stabilised by different ligands or induced by receptor mutations) that have differential capacity for activation of downstream signalling pathways.

The results presented here provide convincing evidence to support LiSS at the GnRH receptor. Furthermore, I have identified a novel signalling pathway, involving activation of SHP-2, which is activated independently of $G_{q/11}$. Delineation of the molecular mechanisms governing LiSS and the signalling proteins activated independently of each other at the GnRH receptor provides valuable information for the development of therapeutics designed to specifically activate desired signalling pathways of the GnRH receptor, without activating other pathways which may induce adverse effects.

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